APOPTOSIS IN BREAST LESIONS

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2000
Oulu, Finland
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Abstract

In this work the extent of apoptosis was studied in a set of 504 benign and malignant breast lesions to elucidate its role in breast tumor development and progression. Also the correlation of apoptosis with estrogen and progesterone receptor positivity, cell proliferation and patients' prognosis was studied. The breast lesions were also analyzed immunohistochemically with antibodies to apoptosis regulating proteins bcl-2 and bax, and caspases 3, 6 and 8. In addition, the immunohistochemical expression of NO\(^+\) synthesizing enzyme iNOS in relation to apoptosis and angiogenesis was studied. Furthermore, the expression of the antioxidative enzyme MnSOD was studied in relation to apoptosis and cell proliferation.

According to the results, the apoptotic index was lowest in benign breast lesions. It was higher in in situ carcinomas, where a gradual increase in the extent of apoptosis from grade I to III in situ carcinoma was seen. The apoptotic index in invasive carcinomas was higher than in in situ carcinomas, and also in invasive carcinomas there was a gradual increase in apoptosis from grade I to III carcinomas. The apoptotic index was highest in recurrent carcinomas.

Strong bcl-2 expression was usually found in benign breast lesions but the immunoreactivity decreased in in situ and invasive carcinomas. There was a significant inverse association between bcl-2 immunoreactivity and the extent of apoptosis. Low bcl-2 immunoreactivity also associated with estrogen- or progesterone receptor negativity. In contrast, bax expression did not show any significant association with apoptosis, hormone receptors or the histologic types of tumors. Strong cytoplasmic caspase 3, 6 and 8 immunoreactivity was found in most carcinomas. It was weaker in in situ carcinomas and only weak immunoreactivity could be seen in benign breast lesions. There was a significant association between the extent of apoptosis and caspase immunoreactivity.

iNOS expression was found in both tumor and stromal cells. iNOS expression in tumor cells was more frequently found in invasive than in in situ carcinomas. Its expression correlated significantly with a high apoptotic index and high vascularization of the lesion. There was significantly less MnSOD immunoreactivity in invasive breast carcinomas compared to in situ carcinomas or benign hyperplasias. MnSOD immunoreactivity did not associate with the extent of apoptosis, but there was a marginal inverse association between cell proliferation and MnSOD expression.

Increased apoptosis was significantly associated with a high cell proliferation, and inversely associated with a positive estrogen status. A high apoptotic index (< 0.50%) was associated with a decreased survival of the patients.

The results of this study show that apoptosis plays a decisive role in the development and progression of breast carcinoma. It is influenced not only by apoptosis regulating proteins, such as bcl-2 and caspases, but also by the estrogen receptor status. Apoptosis was also associated with iNOS positivity, the effect of which is mediated through increased NO\(^+\) production. In line with the suggested role of MnSOD as a tumor suppressor gene, its expression was downregulated in invasive breast carcinoma. In conclusion, the association of apoptosis with patient survival in breast carcinoma may be secondary to its association with tumor cell proliferation and high tumor grade, not necessarily suggesting any causal association between apoptosis and survival.

Keywords: bcl-2, caspases, iNOS, MnSOD.
To Kimmo and Vilma
Acknowledgements

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Oulu, April 2000

Merja Vakkala
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AB-complex</td>
<td>Avidin-biotin-peroxidase-complex</td>
</tr>
<tr>
<td>AEC</td>
<td>Aminoethyl carbazole</td>
</tr>
<tr>
<td>AP%</td>
<td>Apoptotic index</td>
</tr>
<tr>
<td>apaf-1</td>
<td>Apoptosis protease activating factor 1</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bax</td>
<td>Bcl-2 homologous antagonist / killer</td>
</tr>
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<td>bcl-2</td>
<td>B-cell leukemia 2</td>
</tr>
<tr>
<td>BH1-4</td>
<td>Bcl-2 homology domains 1-4</td>
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<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CB</td>
<td>Citrate buffer</td>
</tr>
<tr>
<td>ced</td>
<td>Cell death abnormal</td>
</tr>
<tr>
<td>CMVD</td>
<td>Calculated microvessel density</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic lymphocyte</td>
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<td>CuZnSOD</td>
<td>Copper zinc superoxide dismutase</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
</tr>
<tr>
<td>DED</td>
<td>Death effector domain</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECSOD</td>
<td>Extracellular superoxide dismutase</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
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<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<td>Estrogen receptor</td>
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<td>FADD</td>
<td>Fas associated death domain</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FasR</td>
<td>Fas receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FLICE</td>
<td>Fas ligand interacting cell effector</td>
</tr>
<tr>
<td>FVIII</td>
<td>Factor VIII, von Willenbrand factor</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione peroxidase</td>
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HE
H₂O₂
HPF
Hs
IAP
IkB-α
ICE
Ille
iNOS
JNK
Leu
MEKK-1
MnSOD
NF-κB
NGF
nNOS
NO
NOS
NOS1
NOS2
NOS3
O₂⁻
OH⁺
ONOO⁻
PARP
PBS
PR
RasGAP
Rb
RIP
RNA
ROS
RSR
RT
SREB 1-2
SOD
TBS
TNF
TNF-R
TRADD
TRAF2
TUNEL
Val
•

Haematoxylin-eosin
Hydrogen peroxide
High power field
Histoscore
Inhibitor of apoptosis protein
Inhibitor of NF-κB
Interleukin-1β-converting enzyme
Isoleucine
Inducible nitric oxide synthase
C-Jun N-terminal kinase
Leucine
MEK kinase 1
Manganese superoxide dismutase
Nuclear factor-κB
Nerve growth factor
Neuronal nitric oxide synthase
Nitric oxide
Nitric oxide synthase
Neuronal nitric oxide synthase
Inducible nitric oxide synthase
Endothelial nitric oxide synthase
Superoxide anion
Hydroxyl radical
Peroxynitrite
Poly(ADP-ribose) polymerase
Phosphate buffered saline
Progesterone receptor
Ras GTPase-activating protein
Retinoblastoma
Receptor interacting protein
Ribonucleic acid
Reactive oxygen species
Relative survival rate
Room temperature
Sterol regulatory element-binding proteins 1-2
Superoxide dismutase
Tris buffered saline
Tumor necrosis factor
Tumor necrosis factor receptor
TNF associated death domain
TNF-R associated factor 2
Terminal dUTP nick end labeling
Valine

unpaired electron
List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data is presented.


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1. Introduction

Apoptosis is a biochemically regulated mechanism of the cells to commit suicide (Kerr et al. 1994, Wyllie 1997). It is needed in many physiologic conditions and in embryonic development and may be triggered by several different stimuli (Kerr et al. 1994, Wyllie 1997). In tumor tissue apoptosis is increased, which is probably due to activation of several oncogenes and inactivation of tumor suppressor genes (for a review, see Soini et al. 1998a). The bcl-2 family of proteins serve as critical regulators of pathways involved in apoptosis, either by inhibiting or promoting cell death (Reed 1998). Caspases participate in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a number of vital proteins, resulting in the disassembly and removal of the cell (Nuñez et al. 1998, Thornberry and Lazebnik 1998).

Reactive oxygen species (ROS) are formed continuously in aerobic metabolism. Their increased production causes oxidative stress to tissues, which may lead to cell injury and death (Janssen et al. 1993, Halliwell 1994). Inducible nitric oxide synthase (iNOS) can generate locally high concentrations of nitric oxide (NO*) resulting in a formation of its reactive metabolites (Beckman 1996, Felley-Bosco 1998). These metabolites can cause mutagenesis, nitration of various macromolecules, carcinogenesis and apoptosis (Wink et al. 1998). Antioxidants protect cells by scavenging ROSs, preventing their formation and repairing the damage they have caused (Halliwell 1991). Manganese superoxide dismutase (MnSOD) is considered to be one of the most important antioxidant enzymes against superoxide (Crapo and Tierney 1974).

Breast cancer is the most frequent malignancy among women in Finland as well as in other Western countries (Parkin et al. 1997). Its incidence has been predicted to increase in the future (Engeland et al. 1993). A number of risk factors for breast cancer have been found, however, 50% of women who develop breast cancer have none of them (Lester and Cotran 1999, Madigan et al. 1995). Commonly used indicators for breast cancer patients’ prognosis include the clinical stage of the disease, stromal invasion, histological type and grade (Tavassoli 1992). In addition, factors such as estrogen receptor (ER) and progesterone receptor (PR) status, cell proliferation and c-erbB-2 expression are known to affect prognosis (Tavassoli 1992). Increased apoptosis has been associated with a number of poor prognostic factors, but its prognostic significance in breast carcinoma is still controversial (Berardo et al. 1998, Nishimura et al. 1999, Wu et al. 1999).
The present study was undertaken to investigate the extent of apoptosis in breast carcinoma and its association with biological aggressiveness of the breast lesion, survival and some central prognostic factors, such as cell proliferation and ER and PR status. In addition, the expressions of apoptosis regulating proteins bcl-2, bax, caspase 3, 6 and 8, the expression of NO\(^*\) synthesizing enzyme iNOS, tumor angiogenesis and the expression of the antioxidant enzyme MnSOD were studied.
2. Review of the literature

2.1. Apoptosis

2.1.1. Background

It has been known for almost a century that a balance between cell proliferation and cell death is essential for embryonic development and for homeostasis in adult tissues (Cummings et al. 1997, Granville et al. 1998). The first key finding in the study of cell death was that, in addition to necrosis, another cell death type occurs. “Programmed cell death” in the normally developing mammalian embryo was already well known to developmental biologists in the early 1950s, but few outside the field were aware of its occurrence (Cummings et al. 1997). Studies by Kerr and his co-workers in the 1960s and 1970s proposed evidence of the existence of another cell death distinct from necrosis characterized by small, round cytoplasmic masses and the absence of inflammation (Kerr and Searle 1971, Kerr et al. 1972). “Shrinkage necrosis” was found under both physiologic and pathologic conditions and could be regulated e.g. with hormones. It was in 1972 when the term apoptosis (derived from the Greek word for “falling off”) was first used in this context (Kerr et al. 1972).

Studies of the development of the nematode Caenorhabditis elegans (C. elegans) established the second key finding, that apoptosis involves specific genes and proteins which act within the cells that die. The protein product of ced-9 gene protects cells from apoptosis and products of ced-3 and ced-4 are required for the execution of apoptosis (Horvitz 1999). C. elegans provides a molecular genetic model of cell death (Fig. 1A), which provides a basis for understanding apoptosis in mammals. Vertebrates have evolved entire gene families that resemble C. elegans cell death genes. Caspases are similar to ced-3, the bcl-2 family is related to ced-9, and apoptosis activating factor-1 (apaf-1) and Nod1 are the only mammalian ced-4 homologs reported so far (Yuan et al. 1993, Hengartner and Horvitz 1994, Zou et al. 1997, Inohara et al. 1999) (Fig. 1B).
Fig. 1. A. Apoptosis in *C. Elegans*. B. Apoptosis in mammals. (Modified from Hetts 1998)
2.1.2. Morphology of apoptosis

Apoptosis characteristically affects scattered single cells. The dying cell separates from its neighbors, usually with loss of specialized membrane structures. It undergoes a period of blebbing and contortion. The blebs are membrane-invested extensions of cytosol that are usually devoid of organelles and are reversibly extruded and resorbed. This is followed by compaction and segregation of the nuclear chromatin, with the formation of sharply delineated granular masses that become marginated against the nuclear envelope, and condensation of the cytoplasm. The cell splits into a cluster of membrane bound bodies, each containing a variety of organelles. Apoptotic bodies are ingested by nearby cells and macrophages before they can cause an inflammatory reaction. (Kerr et al. 1972, 1994, Wyllie 1997)

The early events in apoptosis are accomplished quickly, with only a few minutes elapsing between the onset of the process and the formation of apoptotic bodies (Wyllie 1997). Thus, budding cells are rarely observed in tissue sections. The apoptotic bodies are degraded by nearby cells within hours (Kerr et al. 1994, Leist and Nicotera 1997). The duration of the process of apoptotic cell death depends on the stimulus and the cell type. Usually it is estimated to take from 12 to 24 hours (Soini et al. 1998a). Apoptotic cells are visible under microscope for only a few hours (Wyllie 1997).

2.1.3. Apoptosis versus necrosis

Apoptosis is a genetically determined, biologically meaningful, active process that plays a role in physiologic (e.g. retains homeostasis in adult tissues) and pathologic (e.g. in tumors) conditions. Necrosis, in contrast, is an accidental passive process in acute, nonphysiologic injury (e.g. in the center of infarcted tissue). In apoptosis scattered single cells are characteristically affected, not cell groups as in necrosis. In apoptosis cells are rapidly turned into apoptotic bodies, which are then phagocytosed without inflammation. In necrosis, the dying cell swells, cytosolic and nuclear structures alter, the plasma membrane ruptures and internal materials reach the extracellular space inducing an inflammatory reaction. In apoptosis DNA is split into segments that are multiples of approximately 185 bp, whereas in necrosis DNA is broken into randomly sized fragments. In addition, apoptosis is ATP-dependent while necrosis doesn’t require any energy. (Cummings et al. 1997, Hetts 1998, Wyllie 1997)

Necrosis and apoptosis have recently been shown to be mechanically related to each other. It seems that in many pathologic situations apoptosis and necrosis coexist (e.g. in ischemic brain damage areas of necrosis are surrounded by apoptosis) (Leist and Nicotera 1997). Certain stimuli (e.g. heat shock, NO*), depending on dose, can cause both apoptosis and necrosis, and they are both completely blocked with inhibitors of caspases or by overexpressing bcl-2 (Leist and Nicotera 1997).
2.1.4. Induction and inhibition of apoptosis

Some cells are genetically destined for death during the normal development of multicellular organism, or to maintain tissue homeostasis in adult tissue. There may be a universal genetic program for cell death that a variety of stimuli can activate or inhibit. Beyond cell deaths that occur as a programmed feature, apoptotic deaths have been shown to be the result of a variety of extrinsic and intrinsic factors (Hockenbery 1995, Majno and Joris 1995, Soini et al. 1998a). A list of well-known agents which are able to inhibit or to induce apoptosis are listed in Tables 1A and 1B.

2.1.5. Pathways of apoptotic signaling

The extreme heterogeneity of apoptotic triggers is perplexing and suggests that multiple distinct pathways of cell death may lead to similar morphologic changes in dying cells (Hockenbery 1995). Based on the established interactions among the known mediators of apoptosis at least three classic pathways of apoptotic signaling in mammalian cell have emerged. The first one is initiated by the withdrawal of growth factors and is regulated by the bcl-2 family of proteins. This pathway results in cytochrome c release from mitochondria, activation of apaf-1 and triggering of a caspase cascade (Dragovich et al. 1998).

The other well-established apoptosis pathway involves extracellular ligand mediated activation of tumor necrosis factor (TNF) and nerve growth factor (NGF) families, which through adapter molecules can recruit and activate caspases (Kidd 1998, Ashkenazi and Dixit 1998). The best-characterized death receptors are Fas and TNF-R1 (Kidd 1998). For example, the extracellular Fas ligand (FasL) from cytotoxic lymphocyte (CTL) activates Fas receptor (FasR), the intracellular domain of which interacts with a linker molecule called Fas-associated death domain-containing protein (FADD). The apoptotic signal is transduced through the death effector domain (DED) of FADD, which interacts with caspase 8, which can initiate a cascade of caspase activation leading to cell death (Chinnaiyan et al. 1995, Muzio et al. 1996). Another ligand of CTL, TNF, similarly activates TNF-R1 which induces cell death through interaction with a related, but different adapter molecule called TNF-associated death domain protein (TRADD) (Hsu et al 1995). TRADD recruits several signaling molecules to the activated receptor. TNFR-associated factor-2 (TRAF2) and receptor-interacting protein (RIP) stimulate pathways leading to activation of nuclear factor -κB (NF-κB) and c-Jun-N-terminal kinase (JNK), and subsequently inhibition of apoptosis (Hsu et al. 1995, 1996a and 1996b). FADD couples also the TNFR1-TRADD complex to activation of caspase 8 and initiation of apoptosis (Hsu et al. 1996a, Chinnayan et al. 1996). CTL can also activate certain procaspases directly by releasing granzyme B and perforin (Atkinson and Bleackley 1995, Talanian et al. 1997).

One pathway is initiated by DNA damage. Although in part regulated by proteins such as p53, it is not known how this pathway results in caspase activation (Bellamy 1997, Dragovich et al. 1998). In all of these pathways of cell death, bcl-2 family proteins and
caspases play a key role in the regulation and execution of apoptosis (Dragovich et al. 1998).

Table 1A. Inhibitors of apoptosis (Modified from Thompson 1995)

<table>
<thead>
<tr>
<th>Physiologic inhibitors</th>
<th>Viral genes</th>
<th>Pharmacologic agents</th>
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<tr>
<td>Growth factors</td>
<td>Adenovirus E1B</td>
<td>Calpain inhibitors</td>
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<tr>
<td>Extracellular matrix</td>
<td>Baculovirus p35</td>
<td>Cysteine protease inhibitors</td>
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<td>CD40 ligand</td>
<td>Baculovirus IAP</td>
<td></td>
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<tr>
<td>Neutral aminoacids</td>
<td>Cowpox viruscrmA</td>
<td>Tumor promoters:</td>
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<tr>
<td>Zinc</td>
<td>Epstein-Barr virus BHRF1, LMP-1</td>
<td>PMA</td>
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<td>Estrogen</td>
<td>African swine fever virus LMW5-</td>
<td>Phenobarbital</td>
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<td>Androgens</td>
<td>HL</td>
<td>α- hexachloro-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cyclohexane</td>
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<tr>
<td></td>
<td>Herpesvirus γ1, 34.5</td>
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Table 1B. Inducers of apoptosis (Modified from Thompson 1995)

<table>
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<tr>
<th>Physiologic activators</th>
<th>Damage-related inducers</th>
<th>Therapy-associated agents</th>
<th>Toxins</th>
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<tr>
<td>TNF family</td>
<td>Heat shock</td>
<td>Chemotherapeutic drugs</td>
<td>Ethanol</td>
</tr>
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<td>Fas ligand</td>
<td>Viral infections</td>
<td>Gamma radiation</td>
<td>β-amyloid</td>
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<tr>
<td>TNF</td>
<td>Bacterial toxins</td>
<td>UV radiation</td>
<td>peptide</td>
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<tr>
<td>Transforming growth factor β</td>
<td>Oncogenes</td>
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<td></td>
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<tr>
<td>Neurotransmitters</td>
<td>Tumor suppressors</td>
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<td>Glutamate</td>
<td>p53</td>
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<td>Dopamine</td>
<td>Cytolytic T cells</td>
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<td>N-methyl-D-aspartate</td>
<td>Oxidants</td>
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<td>Growth factor withdrawal</td>
<td>Free radicals</td>
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</tr>
<tr>
<td>Loss of matrix attachment</td>
<td>NO*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>Nutrient</td>
<td>deprivation-</td>
<td></td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>antimitabolites</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2. Bcl-2 family

The bcl-2 family is named after its founding member, a protein, overexpressed in human B-cell lymphoma as a result of a t(14;18) chromosomal translocation that causes oncogenesis by suppressing apoptosis (Pegoraro et al. 1984, Tsujimoto et al. 1985, Vaux et al. 1988). Overexpression of bcl-2 protects many cell types against apoptosis in response to such diverse stimuli as cytokine withdrawal, viral infection, hypoxia, ionizing radiation, oxidative stress, p53 or chemotherapeutic drugs (Gajewski and Thompson 1996, Yang and Korsmeyer 1996, Reed 1998).

The bcl-2 family of proteins are mammalian relatives of ced-9 (Vaux et al. 1992, Hengartner and Horvitz 1994). At least 15 bcl-2 family members have been identified in mammalian cells and several others in viruses (Adams and Cory 1998) (Table 2). All members possess at least one of the four bcl-2 homology domains (BH1-BH4) (Adams and Cory 1998). Bcl-2 contains all these four domains and most of the pro-survival members contain at least BH1 and BH2 and some of them also have all four BH domains (Adams and Cory 1998, Reed 1998). Members of the pro-apoptotic bax-subfamily contain BH1-BH3 and resemble bcl-2 very closely (Adams and Cory 1998, Reed 1998). In contrast, pro-apoptotic BH3-subfamily members contain only BH3 domain (Adams and Cory 1998, Reed 1998). Bcl-2 and many other members contain a C-terminal signal-anchor sequence responsible for their position, primarily the outer mitochondrial membrane, nuclear envelope and endoplasmic reticulum (Chen-Levy and Cleary 1990, Krajewski et al. 1993, González-Garcia et al. 1994, Yang et al. 1995a, Zha et al. 1996). Some proteins, like bad, lack this signal-anchor sequence and are cytosolic (Yang et al. 1995b).

Table 2. The bcl-2 family. Three subfamilies are indicated. (Modified from Adams and Cory 1998)

<table>
<thead>
<tr>
<th>Pro-survival: bcl-2 subfamily</th>
<th>Pro-apoptosis: bax-subfamily</th>
<th>Pro-apoptosis: BH3-subfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>Bax</td>
<td>Bik</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>Bak</td>
<td>Blk</td>
</tr>
<tr>
<td>Bcl-w</td>
<td>Bok</td>
<td>Hrk</td>
</tr>
<tr>
<td>Mcl-1</td>
<td></td>
<td>BNIP3</td>
</tr>
<tr>
<td>A1</td>
<td></td>
<td>BimL</td>
</tr>
<tr>
<td>NR-13*</td>
<td></td>
<td>Bad</td>
</tr>
<tr>
<td>BHRF1*</td>
<td></td>
<td>Bid</td>
</tr>
<tr>
<td>LMW5-HEL*</td>
<td></td>
<td>EGL-1*</td>
</tr>
<tr>
<td>ORF16*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KS-Bel-2*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1B-19K*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CED-9*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* non-mammalian homologs
2.2.1. Mechanisms

Members of the bcl-2 family have been shown to homo- or heterodimerize with one another and to titrate one another’s function (Chao et al. 1995, Yang et al. 1995a, Reed 1996). However, heterodimerization is not essential for the function of pro-survival bcl-2 subfamily or pro-apoptotic bax-subfamily (Reed 1998) as earlier indicated (Yin et al. 1994). Though bcl-2 and bax may be capable of functioning independently, dimerization provides an important mechanism for controlling their activity (Reed 1998). For the function of pro-apoptotic BH3 subfamily heterodimerization is essential while they antagonize pro-survival proteins via their BH3 domains (Chittenden et al. 1995, Yang et al. 1995b, Kelekar et al. 1997, Kelekar and Thompson 1998).

Biochemical evidence suggests that the pro-survival proteins may directly inhibit the ced-4-like molecules from activating caspases. Bcl-xI, for example, is able to bind to the ced-4-like portion of apaf-1 and prevent the association of apaf-1 with procaspase 9 followed by caspase 9 activation (Li et al. 1997). Pro-apoptotic members may free apaf-1 from the death inhibitor (Chinnayan et al. 1997, Chaudhary et al. 1998, Hu et al. 1998, Pan et al. 1998).

Pro-survival proteins also seem to maintain organelle integrity (Adams and Cory 1998). For example bcl-2 is able to prevent the release of cytosome c from mitochondria and prevents the cytosome c mediated change in apaf-1 structure to allow procaspase 9 recruitment and processing (Zou et al. 1997, Li et al. 1997). Alternatively bcl-xI blocks apoptosis via interaction with cytosome c function by binding to it and inhibiting its availability in the cytosol (Kharbanda et al. 1997).

In some systems, bcl-2 regulates mitochondrial intracellular calcium levels and the loss of membrane potential produced by proapoptotic stimuli (Granville et al. 1998). Determination of the three-dimensional structure of bcl-xI has revealed that it shares similarities with pore-forming domains of some types of bacterial toxins, prompting the hypothesis that members having BH1 and BH2 domains function by forming pores in organelles such as mitochondria (Muchmore et al. 1996). At least bcl-2, bcl-xI and bax are capable of forming channels with distinct charasteristics (e.g. ion selectivity) (Antonsson et al. 1997, Minn et al. 1997, Schendel et al. 1997, Schlesinger et al. 1997). These proteins may have a membrane transport function that regulates ion flux and protein transport across some of the intracellular membranes to which bcl-2 and its homologs are localized (Krajewski et al. 1993, Reed 1998).

2.3. Caspases

Soon after interleukin-1β-converting enzyme (ICE, now known as caspase 1) was first purified and its cDNA cloned and sequenced, it was found to be related to ced-3, connecting this enzyme to apoptosis and prompting an intense search for other mammalian homologs (Cerretti et al. 1992, Thornberry et al. 1992, Yuan et al. 1993). In 1996 this family of proteins was named caspases (the “c” is intended to reflect a cysteine protease mechanism, and “aspase” refers to their ability to cleave after aspartic acid) followed by an Arabic numeral, which is assigned based on its date of publication.
(Alnemri et al. 1996). To date 14 proteases have been cloned and partially characterized in mammals and some of them have been implicated in the apoptosis process (Nuñez et al. 1998). All caspases share similarities in structure and have near absolute substrate-cleavage specificity for aspartic acid (Asp) residues (Martin and Green 1995, Thornberry and Lazebnik 1998).

Caspases are synthesized as inactive proenzymes (30 to 50 kDa) that contain three domains: an NH$_2$-terminal domain, a large (~20 kDa) and a small subunit (~10 kDa) (Thornberry and Lazebnik 1998). After proteolytic processing by other proteases, often another caspase or granzyme B, or by autocatalytic cleavage, an active form containing both large and small subunits is formed (Darmon et al. 1995, Cohen 1997, Nicholson and Thornberry 1997, Granville et al. 1998). Active caspases are composed of two heterodimers interacting via the small subunits to form a tetramer with two catalytic sites (Walker et al. 1994, Wilson et al. 1994, Rotonda et al. 1996).

Caspases may be grouped into two major subfamilies: an ICE subfamily, comprising caspases 1, 4 and 5 and a ced-3 subfamily, comprising caspases 2, 3, 6, 7, 8, 9 and 10 (Nicholson and Thornberry 1997). The ICE subfamily predominantly plays a role in inflammation, whereas the ced-3 subfamily is involved in apoptosis (Nicholson and Thornberry 1997). In addition, they can be divided according to their structure or the order in the caspase caspase activation cascade (see 2.3.2.) (Alnemri et al. 1996).

### 2.3.1. Proteolytic substrates for caspases

A growing number of caspase substrates have been identified, including enzymes involved in genome function, regulators of cell-cycle progression and structural protein of both the nucleus and cytoskeleton (Nicholson and Thornberry 1997). As a result caspases are able to halt cell-cycle progression (e.g. protein kinase C δ and θ), disable homeostatic and repair mechanisms (e.g. PARP), initiate the detachment of the cell from its surrounding tissue structures (e.g. focal adhesion kinase), disable structural components (e.g. lamin, α-fodrin), mark the dying cell for engulfment by phagocytes (SCREB-1 and 2), activate proapoptotic signaling proteins (e.g. MEKK-1) and inhibit potential survival signals, allowing the death signals to predominate (e.g. IκB-α) (Cohen 1997, Nicholson and Thornberry 1997, Barkett et al. 1997, Cardone et al. 1997). Once caspases are activated, the morphological changes of apoptosis follow, and the process cannot be halted (Hetts 1998).

### 2.3.2. A hypothetical hierarchy of caspases

The hierarchy of caspases has been tentatively assigned based on their different substrate specificity, site of activation in the caspase cascade, different prodomains and phenotypes of knockout animals (Thornberry et al. 1997, Nuñez et al. 1998, Thornberry and Lazebnik 1998). The substrate specificity for caspases 6, 8, 9 and granzyme B [(Ile/Leu/Val)-Glu-X-Asp] corresponds to cleavage sites in effector caspase proenzymes,
suggested that these enzymes play a role upstream of executioner caspases (Thornberry et al. 1997). The specificity of caspases 2, 3 and 7 and ced-3 (Asp-Glu-X-Asp) suggests that these enzymes may function during the effector phase of apoptosis (Thornberry et al. 1997). Caspases 1, 2, 4, 5, 8, 9, 10, 11, 12, 13, have long prodomains, which in certain procaspases physically connect the initiator caspases with regulatory molecules (Nuñez et al. 1998). In contrast, caspases 3, 6, 7 and 14 have short prodomains which do not prefer complex formation with regulatory molecules (Nuñez et al. 1998). According to available information caspases 8, 9 and 10 are proposed to be initiator caspases, and caspases 6, 3 and 7 effector caspases (Thornberry and Lazebnik 1998). The role of caspase 2 is the most uncertain (Thornberry and Lazebnik 1998). The observation that distinct death signals result in the same morphological changes is explained by the finding that effector caspases are activated by different initiator caspases, each of which is activated by a set of proapoptotic signals (Thornberry and Lazebnik 1998). For example, activation of procaspase 8 requires association with FADD through DED (Boldin et al. 1996, Muzio et al. 1996, Hoffman and Tschopp 1997). Caspase 8 then activates an amplifier protease, such as caspase 1, which in turn activates an effector protease, such as caspases 3 and 7 (Fraser and Evan 1996). In the presence of cytochrome c the cofactor apaf-1 binds to and activates procaspase 9 through the caspase recruitment domain which in turn activates caspase 3 (Li et al. 1997). Caspase 10 may be involved in many CrmA-insensitive non-receptor cell deaths, as CrmA poorly inhibits it (Srinivasula et al. 1996). Inhibitors of apoptosis proteins (IAPs), such as survivin, suppress apoptosis through direct caspase and procaspase inhibition (mainly execution caspases 3 and 7) (LaCasse et al. 1998). In granzyme B-mediated apoptosis caspase 10 is processed first and then it cleaves caspases 3 and 7 (Talianian et al. 1997). As caspases 3 and 7 cleave PARP, but not lamins, and caspase 6 cleaves lamins, this suggests that, in cells undergoing apoptosis, activation of caspases 3 and 7 precedes activation of caspase 6 (Cohen 1997).

2.4. Reactive oxygen species

Reactive oxygen species (ROSs) are inevitable products of aerobic metabolism and are formed continuously (Janssen et al. 1993) (Fig. 2). They include hydrogen peroxide (H₂O₂) and free radicals, such as superoxide (O₂⁻), nitric oxide (NO⁻) and hydroxyl radical (OH⁺), which contain one or more unpaired electrons making them more reactive (Bast et al. 1991, Halliwell 1994). ROSs are very reactive metabolites and their increased production causes oxidative stress to tissues, which may cause cell injury and death (Janssen et al. 1993, Halliwell 1994).

2.4.1. NO⁻

NO⁻ is a potent biologic mediator with diverse physiologic and pathophysiologic roles as a vasodilator, neurotransmitter, antimicrobial effector molecule and immunomodulator
(Geller and Billiar 1998). As a free radical, NO$^*$ reacts with other free radicals, molecular oxygen and heavy metals forming metabolites which mediate the biological effects of NO$^*$ (Amb's et al. 1997).

The genotoxicity of NO$^*$ is due to its reaction with either molecular oxygen or superoxide (Felley-Bosco 1998). Still unknown oxidized forms of NO$^*$, possibly such as N$_2$O$_3$, cause DNA base deamination and cross-linking events which can lead to mutagenesis (deRojas-Walker et al. 1995, Tamir et al. 1996, Felley-Bosco 1998). When NO$^*$ and O$_2$$^*$ are formed in close proximity they may form peroxynitrite (ONOO$^*$). Peroxynitrite is a potentially toxic metabolite which can cause nitration of various macromolecules of the cell (Beckman et al. 1990, Beckman 1996). These reactions may also be associated with the activation of carcinogenic nitrosamines, initiation of apoptosis and inhibition of DNA repair enzymes or lipid peroxidation-induced DNA damage (Wink et al. 1998). High concentrations of NO$^*$ also induce wild-type p53 protein accumulation and apoptosis (Amb's et al. 1997).

The effects of NO$^*$ can be tumor promoting or suppressing. High concentration of NO$^*$ can be cytotoxic whereas low concentration may even protect some cell types from damage and apoptosis (Amb's et al. 1997). During the initiation of tumor growth, natural killer cells and macrophages kill tumor cells by a NO$^*$-mediated mechanism (Lala and Orucevic 1998). However, NO$^*$ may also suppress the anti-tumor defense, promote tumor angiogenesis and blood flow in the tumor neovascularature and enhance tumor growth, invasion and metastasis (Lala and Orucevic 1998).

### 2.4.2. NOS

NO$^*$ is synthesized from the amino acid L-arginine by the NO$^*$ synthase (NOS) (Moncada et al. 1991) (Fig. 2). There are three isoforms of NOS: inducible (iNOS, NOS2), neuronal (nNOS, NOS1) and endothelial (eNOS, NOS3), which are all the products of distinct genes. nNOS and eNOS are continuously present in neurons and endothelial cells, respectively. They are both calmodulin-dependent enzymes and generate low levels of NO$^*$ for brief periods of time. (Wink et al. 1998)

#### 2.4.2.1. iNOS

Human chromosome 17cen-q11.2 houses the 26-exon, 37-kb iNOS gene (Nathan and Xie 1994). iNOS is expressed in macrophages, neutrophils, endothelial cells, hepatocytes, cardiac myocytes, chondrocytes and many other cell types (Lala and Orucevic 1998). It is induced by cytokines and can generate locally high concentration of NO$^*$ for prolonged periods of time (Nathan and Xie 1994, Wink et al. 1998).

The presence of iNOS has been investigated in several types of human tumors. In prostate (Klotz et al. 1998), gynecological (Thomssen et al. 1994) and colon carcinomas (Amb's et al. 1998, Kojima et al. 1999) as well as in transitional cell carcinoma of the bladder (Swana et al. 1999) iNOS expression has been found in tumor cells. In
gynecological tumors increased iNOS activity has been shown to be positively correlated with the degree of malignancy (Thomsen et al. 1994).

In breast tumors the identity of NO producing cells is still controversial while iNOS expression has been reported both in stromal cells (Thomsen et al. 1994) and in tumor cells alone (Dueñas-Gonzales et al. 1997, Reveneau et al. 1999). There are no studies on tumor tissues with iNOS in relation to apoptosis.

2.5. Antioxidants

Aerobic organisms have evolved a system of chemical and enzymatic antioxidants which protects cells by scavenging ROSs, preventing their formation and repairing the damage they cause (Halliwell 1991). The antioxidant system includes a number of nonenzymatic antioxidants such as glutathione, α-tocopherol, ascorbate, β-carotene, flavonoids, urate and metal-binding proteins (transferrin, caeruloplasmin, albumin) (Sies 1991, Halliwell 1994). The antioxidants also include three antioxidant enzymes: superoxide dismutases (SODs), catalase (CAT) and peroxidases, of which glutathione peroxidase (GPX) appears to be the most important (Oberley and Oberley 1997). SOD and CAT need no cofactors, but GPX requires several ancillary enzymes, such as glutathione reductase, glutathione S-transferase and glucose-6-phosphate dehydrogenase (Oberley and Oberley 1997) (Fig. 2).

Superoxide dismutases catalyse the dismutation of superoxide to hydrogen peroxide and oxygen (Warner et al. 1991). Three forms of SOD have been described in mammals: mitochondrial manganese superoxide dismutase (MnSOD), cytosolic copper zinc superoxide dismutase (CuZnSOD) and extracellular superoxide dismutase (ECSOD) (Oberley and Oberley 1997).

2.5.1. MnSOD

MnSOD is considered to be one of the most important antioxidant enzymes both against endogenous and exogenous superoxide (Crapo and Tierney 1974, Wong 1995). It is located in the mitochondrial matrix near the electron transport chain, an important cellular source of ROS production within the cell (Warner et al. 1991). The basal expression of MnSOD is usually low, often hardly detectable. The enzyme is induced by a wide variety of factors: cytokines such as tumor necrosis factor (Wong 1988), interleukins and interferon gamma (Tsan et al. 1990), changes in the cellular redox state (Warner et al. 1996) or in response to exposure to various cytotoxic drugs (Akashi et al. 1996, Das et al. 1998).

Although many studies have shown low MnSOD in malignant cells and especially in cells with a high proliferation (Oberley and Oberley 1997), MnSOD is constantly high at least in renal tumors (Oberley et al. 1994), pleural mesothelioma (Kahlos et al. 1998), glioblastomas (Cobbs et al. 1996) and in colon tumors (Janssen et al. 1998). On the other hand, MnSOD is low in prostate carcinomas (Baker et al. 1997). In cell line studies malignant mesothelioma cells were the only ones showing high MnSOD activity.
(Marklund et al. 1982). No previous studies have been conducted on the expression of MnSOD in non-cancerous and cancerous human breast tissues. Epinephrine autooxidation assay has revealed that breast carcinoma tissues contain a variable but usually higher activity of superoxide dismutases than normal breast tissue (Bianchi et al. 1992). A recent study on breast carcinomas showed that a polymorphism in the MnSOD gene, which changes its mitochondrial targeting sequence, increases the risk of breast cancer (Ambrosone et al. 1999).

There are no studies on tumor tissues with MnSOD in relation to apoptosis and proliferation. Previous cell line studies have shown that MnSOD may suppress apoptosis in breast cancer cells (Manna et al. 1998) and IB-3 lung epithelial cells (Zwacka et al. 1998). Experiments with breast cancer cells also suggest that high MnSOD activity reduces cells proliferation (Li et al. 1995, 1998a and 1998b).

![Diagram of ROS production and antioxidant scheme](image)

Fig. 2. Production of ROSs and antioxidant scheme. Abbreviations: SOD = superoxide dismutase, CAT = catalase, GPX = glutathione peroxidase, GSH = reduced glutathione, GSSG = oxidized glutathione, NADP⁺ = nicotinamide adenine dinucleotide phosphate (oxidized), NADPH = nicotinamide adenine dinucleotide phosphate (reduced), G6PD = glucose 6-phosphate dehydrogenase, 6PGD = 6-phosphogluconate dehydrogenase (Modified from Janssen et al. 1993).
2.6. Apoptosis in disease

Abnormal regulation of apoptosis has been implicated in the onset and progression of a growing range of diseases. Most apoptotic disorders feature decreased apoptosis of one type of cell and increased apoptosis of another type of cell (Hetts 1998). For example, in autoimmune diabetes, the primary defect is too little apoptosis of self-reactive T-cells. Secondary cells of the pancreas are destroyed with too much apoptosis (Hetts 1998). Examples of diseases which are associated with abnormal regulation of apoptosis are classified in Table 3 based on their primary dysfunction.

Table 3. Disorders of apoptosis (Modified from Thatte and Dahanukar 1997)

<table>
<thead>
<tr>
<th>Apoptosis is inhibited</th>
<th>Apoptosis is excessive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cancer</strong></td>
<td><strong>AIDS</strong></td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>CD4+ cells</td>
</tr>
<tr>
<td>Glioma</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>Neurodegenerative diseases</td>
</tr>
<tr>
<td>Lymphoid malignancies</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>Amyotrophic lateral sclerosis</td>
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<tr>
<td>Oral squamous cell carcinoma</td>
<td>Parkinsonism</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>Retinitis pigmentosa</td>
</tr>
<tr>
<td><strong>Autoimmune diseases</strong></td>
<td><strong>Epilepsy</strong></td>
</tr>
<tr>
<td>Myasthenia gravis</td>
<td>Alcoholism</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>Brain damage</td>
</tr>
<tr>
<td><strong>Inflammatory diseases</strong></td>
<td><strong>Fetal alcohol syndrome</strong></td>
</tr>
<tr>
<td>Bronchial asthma</td>
<td><strong>Haemopoietic</strong></td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>Aplastic anaemia</td>
</tr>
<tr>
<td>Pulmonary inflammation</td>
<td>CD4+ T lymphocytopenia</td>
</tr>
<tr>
<td><strong>Viral infections</strong></td>
<td><strong>Cooley’s anaemia</strong></td>
</tr>
<tr>
<td>Adenovirus</td>
<td>G6PD deficiency</td>
</tr>
<tr>
<td>Baculovirus</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td>Cowpox</td>
<td><strong>Organ damage</strong></td>
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<tr>
<td></td>
<td>Alcoholic pancreatitis</td>
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<tr>
<td></td>
<td>Cardiac overload</td>
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<tr>
<td></td>
<td>Ischaemic damage (myocardial, retinal, cerebral, renal)</td>
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<td></td>
<td>Keloids</td>
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<td></td>
<td>Long QT syndrome</td>
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<tr>
<td></td>
<td>Obstructive jaundice</td>
</tr>
<tr>
<td></td>
<td>Polycystic kidney</td>
</tr>
</tbody>
</table>
2.6.1. Cancer and apoptosis

Cancer is usually classified as having diminished apoptosis as a primary dysfunction (Hetts 1998, Wyllie 1997). Defective apoptosis permits the persistence of damaged, mutated cells that would otherwise have been deleted (Wyllie 1997). However, apoptosis is generally increased in tumor tissue compared to normal (Soini et al. 1998a). Apoptotic indices are higher compared to normal tissue e.g. in gallbladder (Turunen et al. 2000), salivary gland (Soini et al. 1998c), lung (Törmänen et al. 1999) and colorectal carcinomas (Sinicrope et al. 1996) as well as in oral (Loro et al. 1999a and b) and laryngeal squamous cell carcinomas (Hagedorn et al. 1998). Patients with pulmonary malignancies have also increased amount of alveolar macrophages with apoptotic bodies in their sputum smear compared to healthier patients (Eerola et al. 1998). In contrast, in esophageal and gastric adenocarcinoma the apoptotic indices are lower than in normal or dysplastic tissues (Katada et al. 1997, Hoshi et al. 1998).

Increased apoptosis has been explained by activation of proapoptotic genes (e.g. bax) or cytotoxic T-lymphocytes, mutations in tumor suppressor genes (e.g. p53, Rb), loss of cell adhesion, hypoxia or by increased proliferation (reviewed by Soini et al. 1998a). Diminished apoptosis in cancer could be explained by antiapoptotic IAP called survivin, which is overexpressed in almost all common cancers (Altieri and Marchisio 1999). Dysregulation of matrix metalloproteinases and their inhibitors can also alter normal cell turnover and affect apoptosis in cancer (Baker et al. 1999, Harayama et al. 1999, Li et al. 1999, Mitsiades et al. 1999).

Chemo- and radiation therapy operate primarily through induction of tumor cell apoptosis. It is becoming clear that the process which inhibits apoptosis in some cancer types is also responsible for the therapeutic resistance. (Rudin and Thompson 1997, Rupnow and Knox 1999)

2.7. Methods of detection of apoptosis

Apoptotic cells can be detected in tissue sections because of their characteristic morphological features even in routinely stained sections (Soini et al. 1998a). More refined techniques have also been developed, which are based on the detection of apoptosis specific biochemical changes or expression of apoptosis associated proteins directly on tissue sections (Soini et al. 1998a, Huppertz et al. 1999). For instance, two procedures have been developed that rely on the presence of fragmented DNA in apoptotic cells and allow in situ detection of apoptosis in formalin-fixed, paraffin-embedded material. These are terminal deoxytransferase-mediated bio-dUTP nick-end labeling (TUNEL) which uses terminal transferase to add labeled nucleotides into the 3’-ends of DNA (Gavrielli et al. 1992, Negoescu et al. 1996) and in situ end labeling which uses DNA polymerase to incorporate biotinylated nucleotides at the DNA strand breaks (Wijsman et al. 1993). The labeled DNA is visualized immunohistochemically. Sources of misinterpretations in the end-labeling studies are: not enough field included in the analysis, the magnification used is not high enough, the tumor cells in a given area are counted incorrectly, and variation of morphologically detectable phase of apoptosis. Also
mistakes in the pretreatment and tissue processing as well as the type of labeling enzyme and variations of incubation time may cause false positive and negative results. In cases with DNA damage, autolysis, tissue drying and necrosis, also other cells can become labeled. (Soini et al. 1998a)

A frequently used technique to detect apoptosis is DNA agarose gel electrophoresis, where apoptotic cells show a typical ladder pattern resulting from internucleosomal DNA cleavage, which produces DNA fragments with molecular weights that are multiples of 180 bp (Wyllie 1980). In addition, apoptotic changes can be detected by flow-cytometry, e.g. apoptotic cells produce a reduction in forward light scatter because of their smaller size and reduced DNA content produces a sub-G1(A) peak in the DNA histogram (Ormerod 1998).

The above-mentioned methods detect the last events of apoptosis. Also earlier biochemical events can be visualized and give valuable information about tissue dynamics before apoptotic death. Such markers in the initiation stages are e.g. receptor-ligand interactions (Fas/Fasl, TNF-R1/TNFα), initiator caspases (8, 9, 10) and phosphatidylserine flip from the inner to the outer leaflet of the plasmamembrane, which can all be visualized immunohistochemically, or membrane blebbing visualized by light and electron microscopy (Huppertz et al. 1999). Execution stages can be studied immunohistochemically visualizing regulatory bcl-2 family proteins, execution caspases (3, 6, 7) or caspase substrates (e.g. PARP, lamin B) (Huppertz et al. 1999).

2.8. Benign breast disease

In the female, the complex structure of the breast and its sensitivity to endocrine influences predispose this organ to a number of pathologic conditions. Most diseases of the breast present as palpable masses, inflammatory lesions, nipple secretion, or mammographic abnormalities. Fortunately most of these (90 %) are benign. (Lester and Cotran 1999)

Fibrocystic changes represent the single most common disorder of the breast (Lester and Cotran 1999). The changes seen include gross and microscopic cysts, apocrine metaplasia and blunt duct adenosis (Ellis et al. 1998). Hormonal imbalance is considered to be basis for the development of this disorder (Ellis et al. 1998). In the absence of proliferative breast disease, fibrocystic changes do not elevate the risk of developing cancer (Lester and Cotran 1999).

Some proliferative breast diseases are associated with an increased risk of developing cancer. These are moderate or florid epithelial hyperplasias with or without atypia, sclerosing adenosis and small duct papillomas (Page and Dupont 1990, London et al. 1992). In epithelial hyperplasia cell layers increase due to increased proliferation or diminished apoptosis. Atypical hyperplasias resemble ductal carcinoma in situ. However, the lesions are limited in extent, and the cells are not completely monomorphic in type or fail to fill ductal spaces completely. In sclerosing adenosis there are increased numbers of distorted and compressed acini. Small duct papillomas occur deep within the breast and are lined by a normal two-cell layer. Proliferative lesions are often accompanied by fibrocystic changes. (Lester and Cotran 1999)
2.9. Breast carcinoma

2.9.1. Epidemiology

Breast carcinoma is the most frequent malignancy among women in Finland as well as in other Western countries and it has been predicted to increase in the future (Engeland et al. 1993, Finnish Cancer Registry 1997, Parkin et al. 1997). Also in Africa, South America and Asia breast cancer is among the three most common cancers (Parkin et al. 1997). In Finland over 3000 breast cancer cases appear annually and approximately one of ten women in Finland will develop breast cancer in her lifetime (Finnish Cancer Registry 1997, Finnish Breast Cancer Group 1999). The incidence rate increases with age, but it diminishes after 50 years of age and the average age at diagnosis is 64 years (Engeland et al. 1993). Five-year relative survival rates (RSR) have increased continuously in Finland between 1955-1994 and have reached 80 % among patients diagnosed in 1985-1994 (Dickman et al. 1999).

2.9.2. Etiology

Causes of breast cancer are multifactorial. Several risk factors for breast carcinoma have been found, including long duration of reproductive life, nulliparity or late full-term pregnancy, increasing age, obesity, upper social class, positive family history, increased genetic risk (BRCA1 and 2), previous ionizing radiation and alcohol consumption (Lester and Cotran 1999, Miki et al. 1994, Wooster et al. 1995, Friedenreich et al. 1993). Also earlier proliferative breast disease, carcinoma of the contralateral breast or endometrium are associated with a higher breast cancer risk (Lester and Cotran 1999). However, 50 % of women who develop breast carcinoma have no identifiable risk factors (Madigan et al. 1995).

There are also factors associated with decreased risk of breast carcinomas. They include early menopause and heavy physical activity (Titus-Ernstoff et al. 1998, Ainsworth et al. 1998, Friedenreich et al. 1998).

2.9.3. Diagnostic classification

2.9.3.1. In situ carcinomas

Intraductal carcinoma consists of a malignant population of the cells that spread throughout a ductal system lacking the capacity to invade through the basement membrane (Lester & Cotran 1999). They are divided into five subtypes: comedocarcinoma, solid, cribriform, papillary and micropapillary type (Lester & Cotran 1999). They can further be classified into grades I-III, based on their nuclear grade, the
size of the lesion, margin status and coexisting features, such as the presence or absence of comedo-type necrosis (Elston & Ellis 1998b).

Lobular carcinoma in situ is manifested by proliferation of the cells in terminal ducts or acini. Proliferating cells are monomorphic, loosely cohesive and larger than normal cells. Lobular carcinoma in situ never forms a mass and it is therefore usually an incidental finding. (Lester and Cotran 1999)

2.9.3.2. Invasive carcinomas

The most common type of breast carcinoma is invasive ductal carcinoma (75-80 %). They normally form masses 1 to 2 cm in diameter, rarely exceeding 4 to 5 cm. The tumor consists of malignant cells disposed in cords, solid cell nests, tubules, anastomosing masses, and mixtures of all these invading into stroma. Invasive ductal carcinomas can be graded into three groups: well differentiated (grade I), moderately differentiated (grade II) and poorly differentiated (grade III) carcinoma. The grading is based on three microscopic features: tubular differentiation, the degree of nuclear pleomorphism and the number of mitotic figures. (Elston and Ellis 1998a and b, Lester & Cotran 1999)

Invasive lobular carcinomas cover only 5-10 % of breast carcinomas. They are, however, of particular interest while they tend to be bilateral, multicentric, difficult to detect and form distant metastases more frequently than other subtypes. (Lester & Cotran 1999)

The TNM-classification for breast cancer is shown in Table 4. Breast tumors are classified in Table 5 according to the WHO classification.

2.9.4. Prognostic factors

The most commonly used indicators for prognosis include the clinical stage of the disease (tumor size, lymph node or distant metastases), stromal invasion, histological type, tumor grade and ER and PR status of the primary tumor (Tavassoli 1992). Size of the tumor is a strong predictor of outcome, being positively associated with survival and inversely associated with frequency or time to recurrence (Carter et al. 1989, Quiet et al. 1995). The rare but favorable histologic types of breast cancer include tubular, papillary, mucinous and adenocystic carcinoma (Mansour et al. 1994). Several histologic grading systems have been developed and shown to have prognostic value both in in situ and invasive carcinomas (Ellis et al. 1998, Elston and Ellis 1998b). Positive ER and PR status predicts good prognosis and a high likelihood of response to hormone therapy (Pertschuk et al. 1988, McClelland et al. 1986, Moot et al. 1987). Tumor angiogenesis is a significant prognostic indicator closely linked to metastasis (Horak et al. 1992, Heffelfinger et al. 1996, Kumar et al. 1999). High cell proliferation measured by immunohistochemical labeling (e.g. Ki-67) or by S-phase fraction is also associated with poor prognosis (Joensuu and Toikkanen 1992, Railo et al. 1993, Eissa et al. 1997, Peiró et al. 1997, Railo et al. 1997). Flow cytometry measurements of DNA ploidy are a well
characterized prognostic factor, which distinguishes between diploid and aneuploid tumors, of which aneuploid tumors have a worse prognosis (Mansour et al. 1994).


Table 4. TNM Classification (Sobin & Wittekind 1997)

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumor (T)</td>
<td></td>
</tr>
<tr>
<td>$T_x$</td>
<td>The primary tumor cannot be assessed.</td>
</tr>
<tr>
<td>$T_0$</td>
<td>No evidence of primary tumor.</td>
</tr>
<tr>
<td>$T_{IS}$</td>
<td>Carcinoma in situ: intraductal carcinoma, lobular carcinoma in situ, or Paget’s disease of the nipple with no tumor.</td>
</tr>
<tr>
<td>$T_{1(a-c)}$</td>
<td>Tumor is 2.0 cm or smaller in greatest dimension.</td>
</tr>
<tr>
<td>$T_2$</td>
<td>Tumor is larger than 2.0 cm but not larger than 5.0 cm in greatest dimension.</td>
</tr>
<tr>
<td>$T_3$</td>
<td>Tumor is larger than 5.0 cm in greatest dimension.</td>
</tr>
<tr>
<td>$T_{4(a-d)}$</td>
<td>Tumor of any size with direct extension to chest wall or skin.</td>
</tr>
<tr>
<td>Regional lymph nodes (N)</td>
<td></td>
</tr>
<tr>
<td>$N_x$</td>
<td>Regional lymph nodes cannot be assessed.</td>
</tr>
<tr>
<td>$N_0$</td>
<td>No regional lymph node metastasis.</td>
</tr>
<tr>
<td>$N_1$</td>
<td>Metastasis to movable ipsilateral axillary lymph node(s).</td>
</tr>
<tr>
<td>$N_2$</td>
<td>Metastasis to ipsilateral axillary lymph node(s) fixed to one another or to other structures.</td>
</tr>
<tr>
<td>$N_3$</td>
<td>Metastasis to ipsilateral internal mammary lymph node(s).</td>
</tr>
<tr>
<td>Distant metastasis (M)</td>
<td></td>
</tr>
<tr>
<td>$M_x$</td>
<td>Distant metastasis cannot be assessed.</td>
</tr>
<tr>
<td>$M_0$</td>
<td>No distant metastasis.</td>
</tr>
<tr>
<td>$M_1$</td>
<td>Distant metastasis.</td>
</tr>
</tbody>
</table>
Table 5. Histologic classification of breast tumors (WHO 1981)

<table>
<thead>
<tr>
<th>Type</th>
<th>Benign</th>
<th>Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial tumors</td>
<td>Intraductal papilloma</td>
<td>Noninvasive intraductal carcinoma</td>
</tr>
<tr>
<td></td>
<td>Adenoma of the nipple</td>
<td>Lobular carcinoma \textit{in situ}</td>
</tr>
<tr>
<td></td>
<td>Adenoma Tubular Lactating Others</td>
<td>Invasive invasive ductal carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>invasive ductal carcinoma with a predominant intraductal component</td>
</tr>
<tr>
<td></td>
<td></td>
<td>invasive lobular carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mucinous carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>medullary carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>papillary carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tubular carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>adenoid cystic carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>secretory (juvenile) carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>apocrine carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>carcinoma with metaplasia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>squamous type</td>
</tr>
<tr>
<td></td>
<td></td>
<td>spindle-cell type</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cartilaginous and osseus type</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mixed type</td>
</tr>
<tr>
<td></td>
<td></td>
<td>others</td>
</tr>
<tr>
<td></td>
<td></td>
<td>paget's disease of the nipple</td>
</tr>
<tr>
<td>Mixed connective tissue and epithelial tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miscellaneous tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unclassified tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mammary dysplasia / fibrocystic disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor like lesions</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3. Aims of the study

The specific aims of this study were:

1. To evaluate the extent of apoptosis in benign, premalignant, malignant and metastasized breast lesions

2. To investigate the relation between apoptosis and cell proliferation, estrogen and progesterone receptor status and survival in breast cancer

3. To investigate relations between apoptosis and its regulatory proteins bcl-2, bax, caspases 3, 6 and 8 in breast cancer

4. To find out whether breast cancer cells express iNOS, and its relation to apoptosis and angiogenesis

5. To find out whether breast cancer cells express MnSOD, and its relation to apoptosis and proliferation
4. Materials and methods

4.1. Tumor material in immunohistochemical stainings

The total number of breast lesions studied in this investigation was 504 (451 primary lesions and 53 recurrent carcinomas). The material was collected from the files of the Department of Pathology, Oulu University Hospital and originated from the years 1979 to 1998. The material had been fixed in 10 % buffered formalin and embedded in paraffin consisting of 54 benign lesions, 15 atypical hyperplasias, 145 in situ carcinomas, 207 invasive ductal, 27 invasive lobular and 3 invasive carcinomas of other histological types. The distribution of the cases in different studies is shown in Table 6. The diagnosis was based on a light microscopic evaluation of the cases and was performed by the two pathologists taking part in this study (Y. Soini and P. Pääkkö). The clinical and histopathological characteristics are given in the original papers.

Table 6. Distribution of the cases in studies I-V. Abbreviations: B = benign, ISC = in situ carcinoma, IC = invasive carcinoma, RC = recurrent carcinoma.

<table>
<thead>
<tr>
<th>Dg</th>
<th>Study I N P</th>
<th>Study II N P</th>
<th>Study III N P</th>
<th>Study IV N P</th>
<th>Study V N P</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>26 0</td>
<td>0 0</td>
<td>24 0</td>
<td>0 0</td>
<td>19 0</td>
</tr>
<tr>
<td>ISC</td>
<td>10 0</td>
<td>0 0</td>
<td>74 0</td>
<td>11 32**</td>
<td>50 0</td>
</tr>
<tr>
<td>IC</td>
<td>96 0</td>
<td>77 0</td>
<td>0 82*</td>
<td>18 50**</td>
<td>46 0</td>
</tr>
<tr>
<td>RC</td>
<td>0 0</td>
<td>53 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
</tr>
</tbody>
</table>

N = new cases, P = material from the previous studies *(I, II), **(III)
4.2. Methods

4.2.1. The TUNEL method

In order to detect apoptotic cells the terminal deoxytransferase-mediated dUTP nick-end labeling (TUNEL) method was used. This method is based on 3'-end labeling of apoptotically fragmented DNA by labeled nucleotides which are attached to the fragmented sites by terminal transferase. The TUNEL was performed using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD, USA) in which digoxigenin-labeled nucleotides are used. They can then be detected by anti-digoxigenin antibodies with a normal peroxidase-antiperoxidase reaction. Prior to the TUNEL reaction, the sections were incubated in 20 μg/ml Proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) at room temperature for 15 minutes and in 3 % hydrogen peroxide in PBS, pH 7.2. The color was developed with diaminobenzidine (DAB) after which the slides were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). As a positive control a lymph node with follicular hyperplasia was used.

4.2.1.1. Assessment of the apoptotic index

Evaluation of apoptotic index was performed by two persons independently and a consensus was formed in cases where opinions differed (< 10 % of the cases). In the TUNEL reaction positively labeled apoptotic nuclei and nuclear fragments, e.g. apoptotic bodies, could be detected either singly or in small groups. In the latter case they were usually considered to represent remnants of one apoptotic cell and counted as one apoptotic event. Apoptotic nuclei and single apoptotic bodies were always considered as one apoptotic event. The apoptotic index was determined as the percentage of apoptotic events / the whole tumor cell population. To estimate the apoptotic index (the percentage of apoptotic events in a given area), apoptotic cells and bodies were counted from 10 high power fields (HPFs) with a ×40 objective and this figure was divided by the number of all tumor cells in the same HPFs.

4.2.2. Morphological evaluation of apoptosis

In order to test the reproducibility of estimation of apoptosis by the TUNEL method we also performed apoptosis assessment by light microscopy. The assessment was based on the morphological criteria of apoptosis (Kerr et al. 1994). The morphological apoptosis was assessed from the same tumor samples and the estimation of the apoptotic index was performed in a similar manner as with the TUNEL method.
4.2.3. Immunohistochemical stainings

Five-μm paraffin sections were cut from the specimens and placed either on poly-L-lysine-coated (Sigma Chemicals, St Louis, MO, USA) or on SuperFrost®Plus glass slides (Menzel-gläser, Germany). Sections were soaked in xylene to remove paraffin and rehydrated in graded alcohol series. The endogenous peroxidase was consumed by 0.1 – 3 % hydrogen peroxide in absolute methanol. To prevent non-specific binding either 20% fetal calf serum (FCS) or serum blocking solutions were used. Pretreatments used for enhancing the reactions are listed in Table 8. Primary and secondary antibodies, avidin-biotin-peroxidase (AB-) complexes and chromogens which were used are also listed in Table 8. Blocks with previously determined positivity were used as positive controls. Negative controls were made by replacing the primary antibody with appropriate non-immune sera (I-III) or phosphate buffered saline (PBS) (IV-V) and with preabsorption with the antigenic peptide (polyclonal iNOS in IV).

4.2.3.1. Evaluation of the immunohistochemical staining reactions

The evaluation of the immunohistochemical staining reaction was performed by two persons independently and a consensus was formed in cases where opinions differed (< 10 % of the cases). For bcl-2, bax, caspases 3, 6 and 8, iNOS and MnSOD the intensity and the quantity of the cytoplasmic staining reactions were evaluated separately and a combined score was composed (Table 7).

Table 7A. Evaluation of the intensity and the quantity of the cytoplasmic staining reactions.

<table>
<thead>
<tr>
<th>Intensity</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 = weak</td>
<td>0 = no positivity</td>
</tr>
<tr>
<td>2 = moderate</td>
<td>1 = &lt; 25 %</td>
</tr>
<tr>
<td>3 = strong</td>
<td>2 = 25 – 50 %</td>
</tr>
<tr>
<td>4 = very strong</td>
<td>3 = 50 – 75 %</td>
</tr>
<tr>
<td></td>
<td>4 &gt; 75 %</td>
</tr>
</tbody>
</table>

Table 7B. Division of the combined scores (= Intensity + Quantity) in different works.

<table>
<thead>
<tr>
<th>Bcl-2 (I) and iNOS</th>
<th>Bcl-2 (II) and bax</th>
<th>Caspases 3, 6 and 8</th>
<th>MnSOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = -</td>
<td>0 = -</td>
<td>0 – 2 = +</td>
<td>0 = -</td>
</tr>
<tr>
<td>1 – 2 = +</td>
<td>1 – 4 = +</td>
<td>3 – 5 = ++</td>
<td>1 – 2 = +</td>
</tr>
<tr>
<td>3 – 4 = ++</td>
<td>5 – 8 = ++</td>
<td>6 – 8 = +++</td>
<td>3 – 4 = ++</td>
</tr>
<tr>
<td>5 – 6 = +++</td>
<td></td>
<td></td>
<td>5 – 8 = +++</td>
</tr>
<tr>
<td>7 – 8 = ++++</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In addition to cytoplasmic tumor cell positivity, iNOS staining in stromal cells was also evaluated semiquantitatively and a combined score based on both tumor and stromal cell positivity was composed.

In studies II and III the estrogen and progesterone receptor status was determined semiquantitatively by giving each stained section a histoscore (hs) value based on quantitative and qualitative staining reaction as described previously (Helin et al. 1989). Hs ≥ 100 for ER and hs ≥ 40 for PR were considered positive. In study IV the information about receptor status was collected from the files of Hormone laboratory of Oulu University Hospital.

In study II cell proliferation was expressed as the MIB index, determined as the percentage of MIB positive cells. In study V Ki-67 staining was further evaluated semiquantitatively and divided in four groups: weak = < 5 %, moderate = 5 – 10 %, strong = 10 - 50 % and very strong = > 50 % of cell nuclei positive.

Calculated microvessel density (CMVD) was counted from F VIII-related antigen stained slides on an average of six HFFs with a ×40 objective. In in situ carcinomas both vessels between the neoplastic ducts and a rim of microvessels adjacent to the neoplastic ducts were counted.

### 4.2.4. Immunoblots

In order to test the reliability of the bcl-2 antibody, immunoblot analyses from freshly frozen tissue from two immunohistochemically positive breast carcinoma cases were performed. Tissue samples were homogenized in PBS, centrifuged at 10 000 g for 15 minutes, and samples for electrophoresis were prepared from the supernatants. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed essentially according to Laemmli (Laemmli 1970). Resolved proteins were transferred to nitrocellulose membranes (Towbin et al. 1979). The membranes were incubated overnight at room temperature in TBS (50mM Tris-HCl/200 mM NaCl, pH 7.4) containing 5% dried milk to block non-specific binding sites. The anti bcl-2 antibody (1:250 dilution in TBS) was incubated with the membranes for 60 minutes at room temperature. A biotinated secondary antibody was used, after which the membranes were incubated with streptavidin-horseradish peroxidase complex (ECL Western blotting Kit, Amersham, Buckinghamshire, UK). Between each step, the membranes were washed extensively with TBS containing 0.05% Tween-20. Chemiluminescence-based detection of protein was performed according to the manufacturer’s protocol.

In order to test the specificity of the two iNOS antibodies, immunoblotting analyses using mouse macrophage lysate (Transduction Laboratories, Lexington, KY, USA) were performed. The control macrophages were mixed with the electrophoresis sample buffer and boiled; 75 µg of cell protein was applied to a 12 % sodium dodecyl sulfate-polyacrylamide gel (Laemmli 1970). The gel was electrophoresed for 2.0 h (80 V) at room temperature and transferred onto Hybond ECL nitrocellulose membranes (Amersham, Arlington Heights, IL, USA) in a Mini-PROTEAN II Cell (Bio-Rad, Hercules, CA, USA). The blotted membrane was incubated with the poly- and monoclonal antibodies to iNOS (dilutions 1:2000 with both antibody), followed by
treatment with secondary antibody conjugated to horseradish peroxidase (1:2000, Jackson Immunoresearch Laboratories, Bar Harbor, Maine, USA). The proteins were detected by enhanced chemiluminescence system (Amersham). Cell protein was measured using the Bio-Rad method (Bio-Rad) (Bradford 1976).

4.2.5. Statistical analyses

SPSS for Windows (Chicago, IL, USA) was used for statistical analyses. The significance of the associations was determined using the $\chi^2$ test, Fisher’s exact probability test, correlation analysis and two-tailed t-test. Univariate and multivariate analyses of survival data were undertaken by using survival curves and applying the Kaplan-Meier method with log-rank analysis and the Cox regression model. Probability values $P \leq 0.05$ were considered statistically significant.
Table 8. A list of antigens and respective immunohistochemical methods. Abbreviations: AEC = aminoethyl carbazole, CB = citrate buffer, DAB = 3,3’-diaminobenzidine, EDTA = ethylenediaminetetraacetic acid, RT = room temperature.

<table>
<thead>
<tr>
<th>Antigen (Study)</th>
<th>Pretreatment</th>
<th>Antibody</th>
<th>Dilution Incubation time</th>
<th>Secondary antibody + AB-complex</th>
<th>Chromogen</th>
<th>Staining pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax (II)</td>
<td>CB</td>
<td>Polyclonal rabbit anti-human bax Ab (Pharminen, San Diego, CA, USA)</td>
<td>1:1000 overnight at RT</td>
<td>Antirabbit (Dakopatts, Copenhagen, Denmark) + AB-complex (Dako)</td>
<td>DAB</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Bcl-2 (I, II)</td>
<td>CB</td>
<td>Monoclonal mouse anti-human bcl-2 Ab (clone 124) (Dako A/S, Glostrup, Denmark)</td>
<td>1:50 30 min at RT</td>
<td>Antimouse, (Dakopatts) + AB-complex (Dako)</td>
<td>DAB</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>ER (II-III)</td>
<td>EDTA Buffer</td>
<td>Monoclonal mouse estrogen receptor Ab (Novoceastra Laboratories Ltd, Newcastle upon Tyne, UK)</td>
<td>1:50 60 min at RT</td>
<td>Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA)</td>
<td>DAB</td>
<td>Nuclear</td>
</tr>
<tr>
<td>PR (II-III)</td>
<td>EDTA Buffer</td>
<td>Monoclonal mouse progesterone receptor Ab (Novoceastra Laboratories Ltd.)</td>
<td>1:40 60 min at RT</td>
<td>Vectastain ABC Kit</td>
<td>DAB</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Ki-67 (II)</td>
<td>CB</td>
<td>Monoclonal mouse MIB-1 Ab (Immunotech, Marseilles, France)</td>
<td>1:25 60 min at RT</td>
<td>Antimouse (Dakopatts) + AB-complex (Dako)</td>
<td>DAB</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Ki-67 (V)</td>
<td>CB</td>
<td>Monoclonal mouse anti-human Ki-67 Ab (Zymed, San Francisco CA, USA)</td>
<td>1:50 60 min at RT</td>
<td>Antimouse (Dakopatts) + AB-complex (Dako)</td>
<td>DAB</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Caspase 3 (III)</td>
<td>CB</td>
<td>Polyclonal rabbit CPP32 Ab (Pharminen)</td>
<td>1:500 60 min at RT</td>
<td>Antirabbit (Dakopatts) + AB-complex (Dako)</td>
<td>DAB</td>
<td>Cytoplasmic (granular, nuclear)</td>
</tr>
<tr>
<td>Antigen (Study)</td>
<td>Pretreatment</td>
<td>Antibody</td>
<td>Dilution Incubation time</td>
<td>Secondary antibody + AB-complex</td>
<td>Chromogen</td>
<td>Staining pattern</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
<td>----------</td>
<td>--------------------------</td>
<td>--------------------------------</td>
<td>-----------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Caspase 6 (III)</td>
<td>CB</td>
<td>Goat polyclonal Meh2 Ab (Santa Cruz Biotecnology Inc, Santa Cruz, CA, USA)</td>
<td>1:100 60 min at RT</td>
<td>Antigoat (Dakopatts) + AB-complex (Dako)</td>
<td>DAB</td>
<td>Cytoplasmic (granular)</td>
</tr>
<tr>
<td>Caspase 8 (III)</td>
<td>CB</td>
<td>Goat polyclonal Meh5 Ab (Santa Cruz Biotecnology Inc.)</td>
<td>1:100 60 min at RT</td>
<td>Antigoat (Dakopatts) + AB-complex (Dako)</td>
<td>DAB</td>
<td>Cytoplasmic (granular, membrane bound)</td>
</tr>
<tr>
<td>iNOS (IV)</td>
<td>CD</td>
<td>Polyclonal NOS2 Ab (Santa Cruz Biotecnology Inc.)</td>
<td>1:200 60 min at RT</td>
<td>Histostain-Plus Bulk Kit (Zymed Laboratories Inc., South San Francisco, CA)</td>
<td>AEC</td>
<td>Cytoplasmic, stromal cells</td>
</tr>
<tr>
<td>iNOS (IV)</td>
<td>CB</td>
<td>Monoclonal iNOS Ab (Transduction Laboratories, Lexington, KY, USA)</td>
<td>1:60 60 min at RT</td>
<td>Histostain-Plus Bulk Kit (Zymed)</td>
<td>AEC</td>
<td>Cytoplasmic, stromal cells</td>
</tr>
<tr>
<td>Factor VIII (IV)</td>
<td>0.04% pepsin in 0.01 M HCl</td>
<td>Polyclonal FVIII Ab (Dako A/S)</td>
<td>1:250 30 min at RT</td>
<td>Antirabbit Ab (Dako) + AB-complex (Dako)</td>
<td>DAB</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>MnSOD (V)</td>
<td>-</td>
<td>Polyclonal rabbit anti-human MnSOD Ab (a gift from professor JD Crapo, National Jewish Medical Center, Denver, Colorado)</td>
<td>1:1000 2 h at RT</td>
<td>Histostain-Plus Bulk Kit (Zymed)</td>
<td>AEC</td>
<td>Cytoplasmic</td>
</tr>
</tbody>
</table>
5. Results

5.1. Apoptotic index in breast lesions (I-V)

The average of apoptotic indices in different breast lesions in studies I-V are shown in Fig. 3A. There was a progressive increase in apoptosis from benign lesions (0.14 %) to in situ (0.66 %), invasive (0.97 %) and recurrent carcinomas (1.36 %) (unpublished data).

In study III invasive carcinomas displayed a higher apoptotic index than atypical (P < 0.012) or benign epithelial hyperplasias (P < 0.001). Also in situ carcinomas had a higher apoptotic index than atypical (P = 0.004) or benign epithelial hyperplasias (P < 0.001). In study I there were significantly more cases with a low apoptotic index (≤ 0.22 %) in benign breast lesions and atypical hyperplasia than in carcinomas (P = 0.0002). A statistically significant rise in the apoptotic index was seen between invasive ductal and in situ carcinomas (P < 0.001) and in situ lesions and all invasive carcinomas (P = 0.001) in study IV.

The average apoptotic indices in ductal and lobular in situ carcinomas are graphically shown in Fig. 3B. In studies III and IV there were significantly lower apoptotic indices when comparing grade I to grade II-III lesions (III: P < 0.001 and IV: P = 0.009) or grade I-II to grade III lesions (III: P = 0.012 and IV: P = 0.07).

The apoptotic indices in invasive ductal and lobular and in recurrent breast carcinoma are shown in Fig. 3C. The apoptotic indices increased with the tumor grade. These changes were statistically significant in study IV: grade I and grades II-III (P = 0.02) or grades I-II and grade III (P = 0.01). Invasive lobular carcinomas showed lower apoptotic index than ductal carcinomas.

In study II the average apoptotic index in primary carcinomas was 0.74 %, whereas in the recurrent tumors it was 1.36 % (P = 0.015). The apoptotic index was highest in recurrences in lymph nodes (1.35 %) and in local recurrences in the breast tissue scar (1.31 %), but it was somewhat lower in recurrences at other sites (0.95 %).

In study IV the increase in apoptotic indices from in situ (T1s) (0.70 %) to invasive (T1-4) carcinomas (1.38 %) was statistically significant (P = 0.001). This tendency remained if the apoptotic index in T1-4 (0.83 %) was compared to that of T2-4 (1.44 %) (P = 0.003). In study III the differences in apoptotic index between T1-2 (0.46 %) and T3-4 (0.73 %) and T1-3 (0.50 %) and T4 (0.89 %) were both significant (P = 0.018 and P =
0.015, respectively). In both studies nodal (N) and distant (M) metastases did not associate clearly with apoptosis. (unpublished data)

Fig. 3. A. Apoptosis in different breast lesions. An increase in the apoptotic index can be seen in parallel with the aggressiveness of the breast lesion. B. Apoptotic index in different grades of in situ carcinomas. C. Apoptotic index in different grades of invasive carcinomas. Abbreviations: AP% = apoptotic index, Dg: diagnosis, B = benign, AH = atypical hyperplasia, ISC = in situ carcinoma, IC = invasive carcinoma, RC = recurrent carcinoma, LIS = lobular in situ carcinoma, DCIS I-III = ductal carcinoma in situ grade I-III, ILC = invasive lobular carcinoma, DCI I-III = invasive ductal carcinoma grade I-III
5.1.1. Comparison of the TUNEL method with light microscopy (II, III)

To study the reproducibility of the TUNEL method, we also evaluated the apoptotic index by morphological criteria in invasive breast carcinomas and recurrent tumors and compared the values with those obtained by TUNEL. There was a strong positive correlation between the extent of apoptosis obtained by TUNEL and light microscopic morphology (II: \( r = 0.4498, P < 0.001 \); III: \( r = 0.5018, P < 0.001 \)).

5.1.2. Apoptosis and survival (II)

In study II, with primary and recurrent tumors, patients with a primary tumor showing an apoptotic index of > 0.50 % had a significantly shorter survival (\( P = 0.015 \)). In cases in which the apoptotic index in the recurrent tumor had increased > 0.50 % per year compared to the primary tumor, the survival of the patient was significantly shorter (\( P = 0.009, \) log-rank). By the Cox multivariate regression model, enhanced apoptosis showed a 2.0-fold risk for a shortened survival (\( P = 0.016 \) 95% confidence interval 1.14-3.49) as an independent prognostic factor.

5.2. Bcl-2 (I-II) and bax (II)

In non-neoplastic breast tissue cytoplasmic bcl-2 immunostaining was seen in ductal and acinar cells and weaker reactivity also in myoepithelial cells. The bcl-2 immunoreactivities in different breast lesions are shown in Fig. 4A. In study I there were significantly more cases with high or very high bcl-2 immunostaining scores in benign hyperplasias and sclerosing adenosis (93 %) than in atypical ductal hyperplasias and in situ carcinomas as a group (33 %) (\( P = 0.0008 \)) or invasive carcinomas (42 %) (\( P = 0.0003 \)).

In in situ and invasive carcinomas, there were significantly more cases with a high apoptotic index (> 0.22 %) showing no, low or moderate bcl-2 staining scores than in cases with a low apoptotic index (\( P = 0.01 \)). If all breast lesions (I) were considered the association was very strong (\( P = 0.0004 \)). (I)

Also primary and recurrent tumors with no bcl-2 expression had a significantly higher apoptotic index (1.32 %) than tumors that were positive for bcl-2 (0.71 %)(\( P = 0.010 \)). In recurrent tumors a decrease in bcl-2 immunoreactivity was seen in 4 and an increase in 10 cases. In cases with an increased bcl-2 immunoreactivity in the recurrent tumor, there was an average increase of only 0.15 % in the apoptotic index while in cases with no change or a decrease it was 0.71 % (\( P = 0.10 \)). Patients with tumors showing a strong bcl-2 positivity in the primary lesion had a better prognosis (\( P = 0.02, \) log-rank). (II)

In recurrent tumors a decrease in bax immunoreactivity was seen in 3 cases and an increase in 11 cases. An increase in bax expression was not significantly associated with changes in apoptosis, proliferation or survival. There was no significant correlation between bcl-2 and bax expression. (II)
5.3. Caspases 3, 6 and 8 (III)

Diffuse, weak (+) and inconsistent cytoplasmic positivity was seen for caspases 3, 6 and 8 in non-neoplastic ductal and lobular cells. In breast preneoplastic and neoplastic cells the expression of these caspases was mainly diffuse and intracytoplasmic. Caspases 6 and 8 also expressed granular intracytoplasmic positivity especially in invasive carcinomas, giving an impression of being located in the apoptotic bodies. Caspase 3 had granular and nuclear staining reactions in a few cases. In addition, caspase 8 occasionally expressed membrane bound staining.

The immunoreactivities of the caspases studied are graphically shown in Fig. 4B. The expressions of all three caspases increased in parallel with the histological progression of the breast lesion. There were significantly more cases with increased (+++, ++++) caspase 3 positivity in invasive breast carcinomas (90 %) than in in situ carcinomas (58 %) (P = 0.00002), atypical hyperplasias (25 %) (P = 0.00001) or benign epithelial lesions (22 %) (P = 0.00004). Similarly caspase 6 showed significantly more cases with increased (+++, ++++) expression in invasive carcinomas (87 %) than in in situ carcinomas (60 %) (P = 0.00024), atypical hyperplasias (25 %) (P = 0.00002) or benign epithelial hyperplasias (11 %) (P = 0.00001). With caspase 8, however, invasive carcinomas (83 %) did not show significantly more cases with increased (+++, ++++) positivity than in situ carcinomas (84 %) (P = 0.57) or atypical hyperplasias (57 %) (P = 0.99), but the difference with benign epithelial hyperplasias (22 %) was significant (P = 0.00035). Invasive breast carcinomas showed significantly more cases with strong (+++) caspase 8 positivity than in situ carcinomas (P = 0.029) or atypical hyperplasias (P = 0.0008), and in situ carcinomas showed significantly more cases with strong caspase 8 positive cases than atypical hyperplasias (P = 0.02). Similar associations were found with caspase 3 (P < 0.00001, P = 0.00008, P = 0.057, respectively) and with caspase 6 (P = 0.006, P = 0.00002 and P = 0.12, respectively).

In all cases (III), strong (+++) caspase 3 positivity was significantly associated with increased apoptosis (1.00 ± 1.27 % for strong expression, 0.36 ± 0.56 % for other cases, P = 0.007). A similar association was seen with caspase 6 (1.11 ± 1.21 % vs 0.62 ± 0.99 %, P = 0.012) and caspase 8 (1.06 ± 1.24 % vs 0.53 ± 0.90 %, P = 0.009). In invasive carcinomas, no association with patient survival was observed with caspase 3, 6 or 8. There was also no significant association between strong (+++) caspase 3, 6 or 8 immunoreactivity and a positive estrogen or progesterone receptor status in invasive carcinomas.
5.4. iNOS (IV)

In benign breast epithelial cells inconsistent expression of iNOS could be seen in the ductal and acinar structures. In tumor cells the staining reaction was cytoplasmic. The strongest iNOS expression could be seen in stromal macrophages and neutrophils. Also stromal fibroblasts and endothelial cells often showed cytoplasmic positivity. Forty-six % of the cases showed no staining for iNOS in the tumor cells, 44 % stained weakly (+), 9 % moderately (++) and 1 % strongly (+++). The number of positive stromal cells was low in 37 %, moderate in 41 % and high in 22 % of the cases. There was a clear association between tumor cell positivity and high number of positive stromal cells (P = 0.017). The sum scores for iNOS immunoreactivities were 1 in 22 %, 2 in 56 % and 3 in 23 % of the cases.

In in situ carcinomas the number of cases with iNOS positive tumor cells increased from grade I to III (20 %, 46 % and 73 %). There were significantly less iNOS positive cases in grade I than in grade II-III in situ lesions (P = 0.01) and in grade I-II than in grade III in situ lesions (P = 0.01).

iNOS positivity was more often found in tumor cells of invasive ductal (64 %) than in in situ carcinomas (47 %) (P = 0.05). Also the number of positive stromal cells and sum scores was higher in invasive ductal carcinomas (P = 0.03 and P = 0.004 respectively). All different invasive carcinomas had more cases with a very high number of iNOS positive stromal cells than in situ carcinomas (P = 0.03), and they had also higher sum scores (P = 0.006).
In the whole material tumors with high sum scores (>1) for iNOS had more cases with a higher apoptotic index (≥ 0.63 %) (Fisher’s exact test P = 0.02). Moderate or high number of iNOS positive stromal cells associated also with a higher apoptotic index (P = 0.03) but iNOS positivity in tumor cells alone did not (P = 0.12).

There was no difference in iNOS positivity in tumor cells between invasive (T1a) and in situ (T1b) carcinomas (P = 0.14), but in invasive carcinomas there were more cases with a high number of positive stromal cells (P = 0.05). Also the sum of iNOS positivity in tumor and stromal cells associated clearly with invasive tumors (P = 0.004). A high number of iNOS positive stromal cells associated with nodal and/or distant metastasis (P = 0.05). iNOS positivity in tumor and stromal cells did not associate with estrogen receptor positivity, but it did associate with progesterone receptor positivity (P = 0.02). The iNOS positivity did not correlate with the patients’ survival.

There was a significant association between iNOS positivity in tumor cells and a high (≥12.8) CMVD value (P = 0.04). Also a moderate or high number of positive stromal cells associated with a high CMVD index alone (P = 0.05) and as the sum with iNOS positivity in tumor cells (P = 0.02).

To study the reliability of polyclonal iNOS antibody a part of the cases were evaluated also with a monoclonal antibody. There was a strong positive correlation between iNOS positivities with these two antibodies (P = 0.002).

5.5. MnSOD (V)

Ninety-four % of normal epithelium and 79 % of benign hyperplasias showed positivity for MnSOD. Inconsistent immunoreactivity could be seen in ductal and lobular breast epithelial and myoepithelial cells. In addition, fibroblasts, granulocytes, histiocytes and endothelial cells expressed MnSOD. Positivity was also seen in smooth muscle cells of small vascular structures.

MnSOD positivity in tumor cells could be found in 82 % of the in situ carcinomas and 52 % of the invasive carcinomas. In many cases the stromal cells expressed variable, but usually strong positivity for MnSOD. Invasive carcinomas more often expressed negative staining in tumor cells compared to in situ carcinomas (P = 0.0009) or sections with hyperplasias (P = 0.039) or normal breast epithelium (P = 0.00005). In situ and invasive carcinomas were more often strongly MnSOD positive than hyperplasias (P = 0.034).

There was no association between MnSOD expression and apoptosis in invasive and in situ carcinomas (P = 0.382). There was a marginal inverse association between MnSOD and Ki-67 expression in the carcinomas (P = 0.066).
5.6. Estrogen and progesterone receptors (II)

In study II estrogen receptor negative cases had an apoptotic index of 1.14 %, whereas receptor positive cases had an apoptotic index of 0.68 % (P = 0.042). Progesterone receptor negative cases had a mean apoptotic index of 1.00 %, whereas in those with a progesterone positive status it was 0.66 % (P = 0.16). A loss of positive estrogen or progesterone receptor status in recurrent versus primary tumors could be seen in 6 and 2 cases, respectively. In such cases there was an average increase of 1.39 % in apoptosis, whereas in the other cases it was only 0.41 % (P = 0.17). Cases with a loss in the positive estrogen or progesterone status showed a significant increase in proliferative activity (P = 0.029). Patients with tumors showing a positive estrogen or progesterone receptor status had a better prognosis (P = 0.004 and P = 0.01 respectively, log-rank).

5.7. Cell proliferation (II, V)

In the primary tumors (II) the MIB-index was 7.17 %, and in the recurrent carcinomas it was 11.41 % (P = 0.038). The MIB index was highest in local recurrences (18.72 %) as compared to the primary tumor (P = 0.001). The MIB index in lymph nodes or other sites did not significantly differ from that of primary tumors. There was a positive correlation
between the apoptotic and MIB index \((r = 0.3997, P < 0.001)\). Patients with a high MIB index \((> 7.18\%)\) in the primary tumor did not have a statistically significantly worsened prognosis \((P = 0.10, \text{log-rank})\). In cases in which the MIB index in the recurrent tumor had increased more than 2.00 \% per year compared to the primary tumor, the survival of the patient was significantly shorter \((P = 0.0027, \text{log-rank})\). (II)

Fifty-three \% of the invasive and \textit{in situ} carcinomas showed weak or moderate Ki-67 expression and 47 \% strong expression. Cases (V) with a moderate or strong MIB-index showed significantly more apoptosis (0.82 \%) than cases with a low index (0.33 \%) \((P = 0.003)\). (V)

5.8. Immunoblot analyses (I, IV)

In bcl-2 immunoblot analyses two freshly frozen, immunohistochemically positive breast carcinoma cases and two non-neoplastic breast samples were used. In all of them, a clear positive band corresponding to the 26 kDA molecular weight of bcl-2 protein could be seen.

iNOS immunoblot analyses were done with control macrophages and a positive bands corresponding to the 130 kDa molecular weight of iNOS protein could be detected with both antibodies.
6. Discussion

6.1. Apoptosis in breast lesions

Apoptosis occurs regularly during normal growth and development of the mammary gland. One of the classical examples of apoptosis is reversion of the lactating breast to its resting state after weaning (Kerr et al. 1994). It has become increasingly clear that defective programmed cell death mechanisms contribute significantly to the origin, progression and chemo- or radioresistance of breast cancer, evoking an enormous interest to study apoptosis in breast cancer (Reed 1996).

Analysis of the apoptotic index in the present breast lesions showed a progressive increase in it in parallel with the biological aggressiveness of the lesion. The apoptotic index was lowest in benign lesions, increasing gradually in different grades of in situ and invasive carcinomas. The highest apoptotic index was seen in recurrent tumors.

The results showing an increased apoptosis in more aggressive tumors may seem paradoxical. Similar results have, however, been shown in other tumor types, such as non-Hodgkin’s lymphomas (Soini et al. 1998b) and endometrial carcinomas (Heatley 1995). Recent studies of breast tumors where apoptosis was detected either by morphology (Zhang et al. 1998) or by the TUNEL method (Krüger et al. 1999) also support our notion. The reason for increased apoptosis during tumor progression may be caused by an accumulation and activation of cancer genes, many of which, in addition to influencing tumor cell proliferation, also influence apoptosis. In line with this a significant increase in tumor cell proliferation during breast carcinoma cell progression could also be observed in our material (see 6.5.). Other factors influencing the progressive increase in apoptosis in breast carcinoma are the loss of ER and PR receptors in tumors of a more aggressive nature (Berardo et al. 1998, Nishimura et al. 1999). According to our results there was an inverse association between apoptosis and a positive ER status. Also downregulation of bcl-2 and upregulation of the expression of caspases, aspects which will be described in more detail below, influence the apoptotic index in breast carcinoma.

Other factors which have been reported to associate with increased apoptosis in breast carcinomas include increasing tumor size (Nishimura et al. 1999, Zhang et al. 1998), p53 accumulation (Harn et al. 1997, Berardo et al. 1998, Pillai et al. 1998, Zhang et al. 1998,
Nishimura et al. 1999), aneuploidy (Berardo et al. 1998) and a greater number of lymph node metastases (Berardo et al. 1998, Zhang et al. 1998). In our material increased apoptosis associated also with increasing tumor size (T), but no association between apoptosis and nodal (N) and distant (M) metastasis could be found. In a recent study a low degree of apoptosis was associated with a high histological grade and a high p53 expression in invasive breast carcinomas, but there was no information as to whether the apoptotic index had been calculated in tumor cell population as usual, or whether all cell types had been included (Wu et al. 1999).

In previous studies both increased (Zhang et al. 1998, Nishimura et al. 1999) and decreased (Wu et al. 1999) apoptosis have been linked to shortened survival of the breast cancer patients, or apoptosis has had no correlation with prognosis (Holmqvist et al. 1999). Our studies suggest that an increased apoptosis is associated with a shortened survival of the patients. This notion is supported by the fact that abundant apoptosis is associated with a number of poor prognostic factors (Berardo et al. 1998), a fact which was also seen in our present study. This suggests that the association of apoptosis with a poor prognosis is not necessarily causally related.

In other tumor types enhanced apoptosis is associated with poor survival of the patients in laryngeal (Hirvikoski et al. 1999) and in non-small cell lung carcinoma (Törmänen et al. 1995). In contrast, low apoptosis correlates with poor prognosis at least in oral squamous cell carcinoma of the tongue (Xie et al. 1999) and in cervical cancers (Höckel et al. 1999).

6.2. Bcl-2 and bax

According to the current results, there was a gradual decrease in the bcl-2 expression as the breast lesions became more aggressive. There was also a strong statistically significant inverse association between the expression of bcl-2 and the extent of apoptosis in the whole material studied (I, II), and separately in in situ and invasive carcinomas, reflecting an important antiapoptotic function of this protein in breast lesions. Furthermore, tumors showing a strong bcl-2 expression had a better prognosis than other tumors.

There are reports supporting our findings of diminished bcl-2 expression in preinvasive lesions (Siziopikou et al. 1996) and in invasive carcinomas (Zhang et al. 1997, Zapata et al. 1998) compared to benign or normal breast lesions. This tendency is also seen in oral squamous cell carcinoma (Loro et al. 1999b) and in colorectal adenocarcinoma (Yang et al. 1999). In contrast, bcl-2 is overexpressed in renal cell carcinoma (Huang et al. 1999).

Inverse association between apoptosis and bcl-2 expression in breast tumors has been demonstrated in vitro (Knowlton et al. 1998) and in vivo (Lipponen et al. 1994, Frankfurt et al. 1997, Pillai et al. 1998, van Slooten et al. 1998, Zhang et al. 1998), but there are also opposite results (Berardo et al. 1998). Bcl-2 expression has been studied in different types of benign and malignant lesions in relation to apoptosis. Similarly to breast lesions, also colonic adenomas and carcinomas (Baretton et al. 1996), salivary gland tumors (Soini et al. 1998c) and pancreatic carcinomas (Virkajärvi et al. 1998) show an inverse association between the extent of apoptosis and bcl-2 immunoreactivity. No correlation
between apoptosis and bcl-2 expression is found e.g. in laryngeal (Hirvikoski et al. 1999), ovarian (Diebold et al. 1996) or in non-small cell lung carcinoma (Törmänen et al. 1995).

In contrast to bcl-2, bax did not show any association with apoptosis or prognosis of the patient, even though it was constantly expressed in breast tumors. This notion is supported by a recent study in breast carcinomas with similar results (Rochaix et al. 1999). In other tumor types no association between bax expression and apoptosis is detected in non-small (Törmänen et al. 1999) and large cell lung cancer (Eerola et al. 1999), laryngeal squamous cell carcinomas (Izawa et al. 1999) or in oligodendrogliomas (Delgado et al. 1999). However, in oral squamous cell carcinoma of the tongue (Xie et al. 1999) and in pancreatic carcinoma (Virkajärvi et al. 1998) bax expression is significantly associated with increased apoptosis.

Our results thus suggest that downregulation of bcl-2 expression is a relatively early event in breast carcinoma development and it plays a significant role in steering the inactivation of programmed cell death in breast lesions. In contrast, bax expression did not show any significant correlation with apoptosis. It has to be remembered that it is the ratio of bcl-2 to bax that determines sensitivity to apoptosis, rather than the absolute levels of either protein. In our studies there was no significant association between these two proteins. However, the level of bcl-2 decreased as the breast lesion became more aggressive while bax levels stayed the same. This decrease in the bcl-2:bax ratio probably shifts cells towards apoptosis.

The bcl-2 family of proteins includes at least 15 mammalian homologs (Adams and Cory 1998). In addition to bcl-2 and bax, also antiapoptotic proteins bcl-XL and mcl-1 as well as the pro-apoptotic protein bak have been detected in primary breast tumors in vitro and in vivo (Zapata et al. 1998). Thus, the assessment of bcl-2 and bax alone may fail to give a complete picture of the role of the bcl-2 family in apoptosis regulation.

### 6.3. Estrogen and progesterone receptors

Estrogen receptor positivity was significantly associated with decreased apoptosis, and also progesterone receptor positive cases showed this same tendency. Bcl-2 was also positively associated with hormone receptor positivity, which is in line with other studies (Bhargava et al. 1994, Silvestrini et al. 1994, Hellemans et al. 1995, Lipponen et al. 1995). The results are in keeping with the fact that estrogen stimulation inhibits apoptosis through up-regulation of bcl-2 and a lack of estrogen receptors might thus lead to an increased apoptosis (Wang and Phang 1995). The results also support the previously reported fact that patients with tumors showing a positive estrogen or progesterone receptor status have a better prognosis (Pertschuk et al. 1988, McClelland et al. 1986, Moot et al. 1987).
6.4. Caspases 3, 6 and 8

There was a gradual increase in the expression of caspases 3, 6 and 8 along with the progression of the breast lesion. Moreover, the expression of all these caspases was strongly associated with the rise in the apoptotic index. The results thus indicate an increased synthetic activity of these proteins during neoplastic progression of breast epithelial cells.

There are differences in the expression of caspases in histologically different tumors and tumors of different sites. In colorectal carcinoma the caspase-3 activity is elevated compared to normal mucosa (Leonardos et al. 1999). In leucemic cell lines, the basal mRNA level of the structurally different caspase genes is very heterogenous from one cell line to another and no correlation was observed between them (Droin et al. 1998). In lymphomas caspase 3 expression was increased in the large cell population of large- and mixed-cell follicular non-Hodgkin’s lymphomas and in Reed-Stenber cells of Hodgkin’s disease (Chhanabhai et al. 1997, Krajewski et al. 1997). In pancreatic and gallbladder carcinomas the immunoreactivities of caspases 3, 6 and 8 were found in 70-90 % of the cases without any association with apoptosis (Virkajärvi et al. 1998, Turunen et al. 2000). In neoplastic gastric mucosa the immunoreactivity of caspase 3 was significantly lower than in gastric adenoma or in non-neoplastic mucosa (Hoshi et al. 1998).

The variable expression of caspases and their association to apoptosis might be due to differences in their regulation and in the regulation of apoptosis in tumor cells of different sites in general. Also immunohistochemistry cannot discriminate between the active and inactive forms of the caspases, and only the active form is responsible for the effects of the caspases leading to apoptosis.

6.5. Cell proliferation

There was a significant increase in proliferative activity in recurrent tumors compared with primary tumors. The MIB index was significantly higher in recurrent tumors in the breast scar tissue than in other sites, suggesting that cell populations with a higher proliferative activity have a selective advantage over cells with a lower proliferative capacity in this site. Rather than being due to adhesion molecules, this phenomenon may reflect a selective difference between cell populations in local recurrences compared to recurrences in metastatic sites. In cell populations leading to local recurrences, genetic changes favoring an increased cell proliferation could play a more significant role compared to cell populations with a metastatic phenotype.

Increased cell proliferation associated positively with increased apoptosis in primary and recurrent breast tumors. Several studies like ours have also shown a positive association between apoptosis and cell proliferation in breast tumors (Samoszuk et al. 1996, Berardo et al. 1998, Pillai et al. 1998, Zhang et al. 1998, Krüger et al. 1999, Nishimura et al. 1999). In other tumor types a positive correlation between apoptosis and cell proliferation has been detected at least in bronchial (Törmänen et al. 1999), ovarian (Kupryjanczyk et al. 2000), esophageal (Wang et al. 1999), epidermal squamous cell (Makino et al. 1998) and in colorectal carcinomas (Tatebe et al. 1996) as well as in
6.6. iNOS

While we observed a strong expression of iNOS in stromal macrophages and neutrophils, also 59% of invasive and 47% of in situ lesions displayed iNOS positivity in a proportion of the tumor cells. There was a gradual increase of total iNOS along with the biological aggressiveness of the breast lesions with coexistent increased apoptosis. Tumors with iNOS positivity in tumor and/or stromal cells had also increased vascular density and more nodal and distant metastases. In our material there was no association between iNOS positivity and estrogen receptor positivity. However, the sum of iNOS positivity in tumor and stromal cells correlated with the progesterone receptor positivity.

The published data concerning the identity of NO\(^*\)-producing cells in breast cancer are conflicting. A previous study has demonstrated that in human breast tumors iNOS is mainly expressed in stromal and not in tumor cells, and that its stromal presence correlates with tumor grade (Thomsen et al. 1995). However, in two recent studies strongest iNOS expression has been found in tumor cells correlating with metastatic disease (Dueñas-Gonzales et al. 1997) and progesterone receptor positivity (Reveneau et al. 1999), supporting our findings. Also a study with ZR-75-1 human breast cancer cells revealed that these cells contain iNOS and produce NO\(^*\) spontaneously (Alalami and Martin 1998).

Increased production of NO\(^*\) by iNOS in breast tumor and stromal cells could be one reason for accelerated apoptosis in breast carcinoma. Previous in vitro studies have reported evidence both for and against proapoptotic influence of NO\(^*\) depending on cell type and NO\(^*\) concentration. It has been shown to inhibit apoptosis in endothelial cells (Dimmel er et al. 1997), hepatocytes (Kim et al. 1997), lymphocytes (Mannick et al. 1994), leukocytes (Mannick et al. 1997) and eosinophils (Beavais et al. 1995). NO\(^*\) induces apoptosis in macrophages (Sarih et al. 1993), pancreatic \(\beta\)-cells (Kaneto et al. 1995) and thymocytes (Hortelano et al. 1997). There is also a study suggesting that low concentrations of NO\(^*\) inhibit apoptosis, but high concentrations of NO\(^*\) induce apoptosis in human venous endothelial cells (Shen et al. 1998).

NO\(^*\) may also be an additional factor participating in the progression of breast carcinoma, by enhancing tumor growth, invasiveness and metastatic ability. A part of this effect might be mediated through an increased angiogenesis. In vitro NO\(^*\) increases the growth of coronary venular endothelial cells (Ziche et al. 1994). The ability of NO\(^*\) to potentiate angiogenesis is seen also in in vivo studies on rabbit (Ziche et al. 1994) and rat
(Leibovich et al. 1994) cornea and in a murine mammary tumor model (Jadeski & Lala 1999). Increased angiogenesis would enhance the nutrition of the tumor cells and would make more blood vessels available for tumor cells to invade.

6.7. MnSOD

MnSOD expression was less frequent in neoplastic epithelial cells of invasive breast carcinomas than in preinvasive or non-neoplastic breast epithelium. There were no quantitative differences in the MnSOD expression between in situ carcinomas and non-neoplastic epithelium. On the other hand, carcinoma cases more often showed stronger staining in tumor cells than benign epithelial lesions. A previous study suggesting that breast carcinoma tissues contain a variable but usually higher activity of superoxide dismutases than normal breast tissue (Bianchi et al. 1992) is not contradictory to our result, while breast tumor tissues contain MnSOD positive reactive non-neoplastic cells, which may increase the total amount of MnSOD in tumor tissues.

Decreased MnSOD expression in invasive compared to in situ carcinomas or nonmalignant epithelial cells suggests that a decreased MnSOD expression is associated with the development of an invasive phenotype of the neoplastic breast epithelial cells. This is in line with the suggestion that MnSOD might operate as a tumor suppressor gene in breast cancer and inhibit tumor cell motility, invasion and proliferation (Li et al. 1995, 1998a and 1998b).

There are no studies on breast tumor tissues with MnSOD in relation to apoptosis and proliferation. Previous in vitro studies have suggested that in breast cancer cells high MnSOD activity reduces cell proliferation and apoptosis (Li et al. 1998a and 1998b, Manna et al. 1998, Zwacka et al. 1998) These results cannot, however, be extrapolated to the situation in vivo. According to the present results MnSOD expression might abrogate proliferation in breast carcinomas also in vivo. On the other hand, breast carcinomas did not show any significant association between spontaneous apoptosis and MnSOD expression. Consequently, in breast carcinomas the influence of MnSOD on proliferation might be more important for tumor evolution than its influence on apoptosis.
7. Summary and conclusions

In this study, the extent of apoptosis in benign, premalignant and malignant lesions was studied with the TUNEL method. Apoptosis was also correlated with some prognostic factors, survival of the patients and some known regulatory proteins of apoptosis. In addition, the ability of breast tumor cells to produce and scavenge ROSs was studied in relation to apoptosis.

It was shown that the apoptotic index in breast tumors increases in parallel with the biological aggressiveness of the breast lesion. Increased apoptosis correlated with the known poor prognostic factors such as ER negativity and high cell proliferation. Increased apoptosis was associated with the patients’ decreased survival in breast carcinoma, which may be secondary to its association with high tumor grade, increased cell proliferation and hormone reseptor negativity, not necessarily suggesting any causal association between apoptosis and survival.

The antiapoptotic nature of the bcl-2 protein was clearly seen as its expression decreased in significant inverse association with apoptotic index. The changes in bax expression were less clear, but a decrease in the bcl-2:bax ratio probably shifts breast tumor cells towards apoptosis.

Caspase 8 plays a central role in the signaling of apoptosis after Fas and TNFR1 activation. Caspases 3 and 6 are effector caspases suggested to act downstream of caspase 8. All these caspases were up-regulated in parallel with the apoptotic index supporting this hypothesis.

iNOS expression could be seen in both tumor and stromal cells of breast carcinoma, suggesting that they are able to modulate NO production. iNOS positivity increased from \textit{in situ} to invasive carcinomas and it correlated with increased apoptosis and vascularization. Increased production of iNOS might thus be one additional factor participating in the induction of apoptosis. It might also enhance tumor progression by increased angiogenesis.

MnSOD levels diminished from benign to \textit{in situ} and invasive carcinomas, exposing tumor tissues to oxidative stress. According to this work the influence of MnSOD on proliferation might be more important for tumor evolution than its influence on apoptosis in breast carcinoma.

The results of this study show that apoptosis is increased in parallel with the neoplastic progression of the breast lesions. Apoptosis in breast lesions is also regulated by some
intrinsic factors, such as bcl-2, caspases and iNOS. In conclusion, apoptosis plays a
decisive role in the development, progression and prognosis of breast carcinoma.
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Original articles

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The extent of apoptosis is inversely associated with bcl-2 expression in premalignant and malignant breast lesions

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The extent of apoptosis is inversely associated with bcl-2 expression in premalignant and malignant breast lesions

Aims: In this study we investigated the extent of apoptosis in benign, premalignant and malignant breast lesions and its association with the immunohistochemical expression of bcl-2 oncoprotein.

Methods and results: In order to detect apoptotic cells and bodies in tissue sections, the 3'-end DNA labelling method was used. Immunohistochemical staining was performed by using the avidin–biotin–peroxidase complex technique. A monoclonal antibody against bcl-2 oncoprotein was used and the specificity of the antibody was confirmed by immunoblot analysis. According to the results the extent of apoptosis, as determined by the apoptotic index, was lowest in benign ductal hyperplasias and sclerosing adenomas (0.15% and 0.07%, respectively). It was moderately elevated in atypical hyperplasias and in-situ carcinomas (0.20% and 0.40%, respectively) and highest in invasive carcinomas (0.76%). In ductal invasive carcinomas, grade I lesions showed a lower apoptotic index (0.52%) than grade II (0.72%) and grade III (1.17%) carcinomas. The apoptotic index was not significantly lower in lobular (0.82%) than in ductal invasive carcinomas (0.85%). bcl-2 immunohistochemistry was inversely related to the apoptotic index. In all cases studied the inverse association was very strong (P = 0.0004) but it was also present when only carcinomas were analysed (P = 0.01). In benign and atypical hyperplasias, bcl-2 positivity was observed in all cases, but such cases were less frequent in in-situ lesions and in invasive carcinomas.

Conclusions: The results show that there is an inverse relationship between the extent of apoptosis and bcl-2 expression in breast lesions suggesting that its expression affects the regulation of apoptosis in them.

Keywords: apoptosis, bcl-2, breast, carcinoma

Introduction

Apoptosis is a biochemically regulated mechanism of the cells to commit suicide. It is needed in many physiological conditions and in embryonic development and may be triggered by several different stimuli. In hormone-dependent tissues, such as prostate or breast tissue, withdrawal of the hormonal stimulus causes apoptosis in the affected cells. Glucocorticoids trigger apoptosis in thymic lymphoid cells, also radiation and cytostatic drugs may trigger apoptosis.

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In tumour tissue apoptosis is increased. This is probably due to the activation of several oncogenes and inactivation of tumour suppressor genes which affect apoptosis. Examples of such genes are bcl-2 and its related genes, p53, Rb and c-myc. bcl-2 is a proto-oncogene which is able to inhibit apoptosis. It encodes a 26 kDa protein, which can be found on mitochondrial membranes, the nuclear envelope and endoplasmic reticulum. Due to its translocation to an active promoter site, it is activated in human follicular lymphomas which leads to a clonal expansion of the cells because they are unable to enter apoptosis.

There are several bcl-2-related genes such as bad.
bad, bax, bcl-xL, bcl-xS and mcl-1 which may affect apoptosis. The corresponding proteins are able to dimerize with each other. Such a dimerization is important for the function of these proteins. An over-representation of the bcl-2/bax heterodimers, for instance, leads to a decreased apoptosis while over-representation of bax/bax homodimers favors apoptosis. bcl-2 expression has previously been studied in breast carcinomas. It has been shown that its expression is related to a favorable prognosis, but is not an indicator of the prognosis of breast cancer patients. In node positive breast carcinomas, however, bcl-2 expression is independently related to a better prognosis. Strong bcl-2 expression is also associated with a positive oestrogen or progesterone receptor status. The expression of bcl-2 in relation to apoptosis, as determined by 3′-end DNA labelling, has not previously, however, been studied in preinvasive or invasive lesions. This study was undertaken to test the hypothesis that the extent of apoptosis in premalignant and malignant breast lesions might depend on the type and grade of the lesion, and that the expression of bcl-2 oncoprotein might be related to the extent of apoptosis in these lesions.

Materials and methods

TUMOUR MATERIAL

A total of 132 cases of benign, premalignant and malignant breast lesions consisting of 11 benign ductal hyperplasias, three sclerosing adenoses, 12 atypical ductal hyperplasias (two lobular, 10 ductal), 10 in-situ carcinomas (two lobular and eight ductal consisting of five low-grade cribriform and three high-grade comedo-type lesions), 17 lobular invasive and 79 ductal invasive carcinomas were studied. The material was collected from the files of the Department of Pathology, Oulu University Hospital. The material had been fixed in neutral formalin and embedded in paraffin. The diagnosis of all the cases was based on a light microscopic examination using the conventional haematoxylin and eosin (H & E) stain. The grading of the ductal invasive carcinomas was performed by the two pathologists taking part in this study (YS and PP) according to the criteria of Bloom and Richardson. In ductal carcinomas, there were 18 grade I, 31 grade II and 30 grade III tumours.

3′-END LABELLING OF DNA IN APOPTOTIC CELLS

In order to detect apoptotic cells, in-situ labelling of the 3′-ends of the DNA fragments generated by apoptosis-associated endonucleases was used. The 3′-end labelling of DNA was performed using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) as previously described. The sections, after being dewaxed in xylene and rehydrated in alcohol, were incubated with 20 µg/ml Proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) at room temperature for 15 min. The endogenous peroxidase activity was blocked by incubating the slides in 2% hydrogen peroxide in PBS, pH 7.2. The slides were then treated with terminal transferase enzyme and digoxigenin-labelled nucleotides after which antidigoxigenin-peroxidase solution was applied on the slides. The colour was developed with diaminobenzidine after which the slides were lightly counterstained with haematoxylin. For control purposes we used tissue sections from hyperplastic lymph nodes showing an increased number of apoptotic cells within germinal centres.

ASSESSMENT OF THE APOPTOTIC INDEX

Cells were defined as apoptotic if the whole nuclear area of the cell labelled positively. Apoptotic bodies were defined as small positively labelled globular bodies in the cytoplasm of the tumour cells which could be found either singly or in groups. The apoptotic index was defined as a sum of the apoptotic cells and bodies and it reflected the total number of apoptotic events in a given area. When many apoptotic bodies were found in a group but clearly located in one cell, the group of apoptotic bodies was counted as one. To estimate the percentage of apoptotic events in a given area, i.e. the apoptotic index, the number of tumour cells in 10 high-power fields (HPF) areas (objective × 40; diameter of the field, 400 µm) was also calculated and the number of apoptotic events in the same fields was then devided by this figure thus giving the percentage of apoptotic events per cell population. The percentage of apoptotic cells was obtained in a similar manner.

IMMUNOHISTOCHEMICAL STAINING

Five-µm sections were cut from the specimens and placed on poly-L-lysine-coated (Sigma Chemicals, St Louis, MO) glass slides, air-dried overnight and processed further within a few days. They were dewaxed in xylene and rehydrated in graded alcohol. Endogenous peroxidase was blocked by immersing the sections in 0.1% hydrogen peroxide in absolute methanol for 20 min. Non-specific binding was blocked by incubating the slides in 20% fetal calf serum in PBS for 20 min.

A monoclonal antibody (clone 124) against bcl-2 oncoprotein was obtained from Dako (Glostrup, Denmark). Before application of the primary antibody, the
Table 1. The percentage of apoptotic cells, the apoptotic index and bcl-2 immunohistochemistry in different breast lesions

<table>
<thead>
<tr>
<th>Number of</th>
<th>Percentage of apoptotic cells</th>
<th>Apoptotic index</th>
<th>bcl-2 immunohistochemistry (staining scores)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cases</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Ductal</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>hyperplasia</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Sclerosing</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>adenosis</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Atypical</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>hyperplasia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-situ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High grade</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Low grade</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Lobular</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>carcinoma</td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Ductal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carcinoma</td>
<td></td>
<td></td>
<td>39</td>
</tr>
</tbody>
</table>

*bcl-2 immunohistochemistry not performed in two cases due to exhaustion of the tissue blocks.

sections were heated in a microwave oven in 10 mM citric acid monohydrate, pH 6.0, for 5 min.

After an overnight incubation with the primary antibody (dilution 1:50), a biotinylated secondary antiserum antibody (Dakopatts, Copenhagen, Denmark) was applied (dilution 1:300 for both) followed by the avidin—biotin—peroxidase complex (Dakopatts). The colour was developed by diaminobenzidine, whereafter the sections were lightly counterstained with haematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany).

Negative control staining was carried out by substituting non-immune mouse serum for the primary antibody. As a positive control for bcl-2 immunostaining we used tissue sections from a lymph node with follicular hyperplasia.

The intensity of the bcl-2 immunostaining was evaluated by dividing the cytoplasmic staining reaction in four groups: I = weak cytoplasmic staining intensity, 2 = moderate cytoplasmic staining intensity, 3 = strong cytoplasmic staining intensity, and 4 = very strong cytoplasmic staining intensity. The proportion of the immunostaining was evaluated as follows: 0 = no positive immunostaining, 1 = < 25% of tumour cells showing cytoplasmic positivity, 2 = 25–50% of tumour cells showing cytoplasmic positivity, 3 = 50–75% of tumour cells showing cytoplasmic positivity, and 4 ≥ 75% of tumour cells showing cytoplasmic positivity.

A combined score for bcl-2 immunostaining, based on both qualitative and quantitative immunostaining was composed by adding the qualitative to the quantitative score. This sum score was then divided in five groups as follows: − = score 0, + = score 1–2, ++ = score 3–4, +++ = score 5–6, and ++++ = score 7–8.

**IMMUNOBLOT ANALYSIS**

Tissue samples were homogenized in phosphate-buffered saline, centrifuged at 10 000 g for 15 min, and samples for electrophoresis were prepared from the supernatants. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis was performed essentially according to Laemmli.43. Resolved proteins were transferred to nitrocellulose membranes. The membranes were incubated overnight at room temperature in TBS (50 mM Tris-HCl/200 mM NaCl, pH 7.4) containing 5% dried milk to block non-specific binding sites. The anti-bcl-2 antibody (1:250 dilution in TBS) was incubated with the membranes for 60 min at room temperature. A biotinylated secondary antibody was used, after which
Table 2. The distribution of the apoptotic index in different histological breast lesions

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Apoptotic index</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤.0.22%</td>
<td>&gt;0.22%</td>
</tr>
<tr>
<td>Benign hyperplasia</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Sclerosing adenosis</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Atypical hyperplasia</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Lobular invasive</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Ductal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Grade II</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Grade III</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td>73</td>
</tr>
</tbody>
</table>

the membranes were incubated with streptavidin–horseradish peroxidase complex (ECL Western blotting Kit, Amersham, Buckinghamshire, UK). Between each step, the membranes were washed extensively with TBS containing 0.05% Tween-20. Chemiluminescence-based detection of proteins was performed according to the manufacturer’s protocol.

Results

The results are compiled in Tables 1–3. The apoptotic index was lowest in benign ductal hyperplasia and sclerosing adenosis. It progressively increased in atypical ductal hyperplasia and in intraductal carcinoma and was highest in invasive carcinomas (Figures 1–3). In invasive ductal carcinomas the apoptotic index was highest in grade III and lowest in grade I tumours. The mean apoptotic index in invasive carcinomas was 0.76%.

There were significantly more cases with a low apoptotic index (≤0.22%) in benign breast lesions and atypical hyperplasias than in carcinomas (P = 0.0002, Fisher’s exact test). No significant difference in apoptosis was, however, observed between intraductal and invasive carcinomas (P = 0.28, Fisher’s exact test), invasive lobular and ductal (P = 0.61, Fisher’s exact test) or grade 1 and grade II–III carcinomas (P = 0.88, Fisher’s exact test). In high-grade comedo-type in-situ carcinomas the apoptotic index was considerably higher than in low-grade

Table 3. Apoptotic index and bcl-2 immunostaining in in-situ and invasive carcinomas

<table>
<thead>
<tr>
<th>bcl-2 immunoreactivity</th>
<th>Apoptotic index (number of cases)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤0.22%</td>
<td>&gt;0.22%</td>
</tr>
<tr>
<td>−, +, ++</td>
<td>18</td>
<td>48</td>
</tr>
<tr>
<td>++++, +++</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>68</td>
</tr>
</tbody>
</table>

P = 0.01, Fisher’s exact test.
cribriform lesions, but due to a small number of cases a reliable statistical analysis could not be performed.

In non-neoplastic breast tissue bcl-2 immunostaining was seen in ductal and acinar cells. Ductal epithelial cells many times stained stronger than the acinar cells. The bcl-2 staining in myoepithelial cells was weaker. Apocrine metaplastic cells did not stain positively for bcl-2. In bcl-2 immunostaining almost all cases of benign ductal hyperplasia and sclerosing adenosis showed high or very high bcl-2 staining scores. In atypical hyperplasias there was a slight decrease in the quantity of bcl-2 immunostaining scores. Many of the in situ and invasive carcinomas showed low or moderate bcl-2 immunostaining scores (Figures 4 & 5).

There were significantly more cases with high or very high bcl-2 immunostaining scores in benign or atypical hyperplasias and sclerosing adenoses than in in-situ or invasive carcinomas \( (P = 0.007, \text{ Fisher’s exact test}). \) If atypical hyperplasias were included in the carcinoma group the statistical significance was even stronger \( (P = 0.0001, \text{ Fisher’s exact test}). \) If benign ductal hyperplasias and sclerosing adenosis were compared with atypical ductal hyperplasias and in-situ carcinomas as a group, or invasive carcinomas as a group, the difference between the statistical significance in bcl-2 staining was about the same \( (P = 0.0008 \text{ and } P = 0.0003, \text{ respectively, Fisher’s exact test}). \) In line with this, there was no statistically significant difference between atypical hyperplasias and in-situ carcinomas and invasive carcinomas in bcl-2 immunostaining \( (P = 0.37, \text{ Fisher’s exact test}). \) Also, no significant difference was observed between lobular and ductal carcinomas \( (P = 0.10, \text{ Fisher’s exact test}). \) in bcl-2 staining. The difference between grade I and grade II–III invasive ductal carcinomas or between grade I and III tumours in bcl-2 staining was almost statistically significant \( (P = 0.08 \text{ and } P = 0.06, \text{ respectively, Fisher’s exact test}). \)

In in-situ and invasive carcinomas, there were significantly more cases with a high apoptotic index \( (> 0.22\%). \) showing no, low or moderate bcl-2 staining scores than in cases with a low apoptotic index \( (P = 0.01, \text{ Fisher’s exact test}) \) (Table 3). If all cases were considered, the association was very strong \( (P = 0.0004, \text{ Fisher’s exact test}) \).
Our results are generally in line with a previous study of Sziöölő et al. who found a diminished bcl-2 immunoreactivity in ductal in situ carcinomas and in invasive breast carcinomas\textsuperscript{10}. In contrast to their results we also found a slight diminution in bcl-2 immunoreactivity already in atypical hyperplasias. The difference may depend partly on our scoring system of bcl-2 immunoreactivity where qualitative changes in bcl-2 immunoreactivity were also stressed. Our results thus suggest that downregulation of bcl-2 expression is a relatively early event in breast carcinoma development. This is reflected by the fact that in our material only 30% of in situ carcinomas show high or very high bcl-2 staining scores, a proportion which was similar to that in the invasive carcinomas.

The decrease in bcl-2 immunoreactivity in pre-invasive breast lesions is in contrast to that found in some other pre-invasive lesions, such as gastrointestinal or skin epidermal dysplasias where an increased bcl-2 expression has been found\textsuperscript{17,28}. One reason for this might be the hormonal sensitivity of the breast epithelial cells. It is known that bcl-2 expression in normal breast epithelial cells is hormone-dependent and that a high bcl-2 expression prolongs the survival of hormone dependent epithelial cells\textsuperscript{29}. It has also been shown that there is a strong association between a positive oestrogen and progesterone receptor status and bcl-2 expression in breast carcinomas\textsuperscript{15,18}. It may thus be that in a subpopulation of the pre-invasive lesions there is a gradual loss of oestrogen and progesterone receptors which would be reflected in the decreased bcl-2 positivity.

There are several studies on the extent of apoptosis in various epithelial and non-epithelial tumours\textsuperscript{9-11,17,28}. The extent of apoptosis varies and is high in non-small cell and small cell lung carcinomas and colorectal carcinomas, for instance, where the mean apoptotic index, as judged by the 3'-end DNA labelling, is 1.2%, 2.3% and 1.9%, respectively\textsuperscript{9,10,27}. Our results in invasive breast carcinomas show an average apoptotic index of 0.76% which is lower than in these tumour groups. To our knowledge, there are no previous studies on apoptosis in breast carcinomas using the 3'-end DNA end labelling method as a detection system. In a previous study by Lipponen et al. where apoptosis was determined morphologically by light microscopy, the authors used a different type of assessment for the apoptotic index making it hard to compare our results with the extent of apoptosis in their material\textsuperscript{30}.

bcl-2 expression has been studied in several different types of epithelial malignancies\textsuperscript{9-11,16-19,31-33}. There is a high bcl-2 expression in breast and prostate carcinomas while lung and hepatocellular carcinomas
show less positivity9–11,16–19,32. In our study 66 out of 106 carcinomas (62%) expressed bcl-2 positivity which is in line with the previous studies16,19. In breast carcinomas, no significant difference could be seen in the apoptotic index between ductal and lobular carcinomas. There was also no significant difference in the extent of bcl-2 immunoreactivity between these tumours. In spite of the fact that grade I ductal adenocarcinomas showed less apoptosis than grade II and grade III tumours, there was no significant difference in bcl-2 immunoreactivity between tumour of different grade, even though grade I tumours tended to have more bcl-2-positive cases. In some previous studies low-grade tumours expressed more bcl-2 than high-grade tumours while in some other studies no difference was found16,19.

In conclusion, our results show that there is a gradual increase in the extent of apoptosis and a decrease in bcl-2 expression in breast lesions as they become histologically more aggressive. The statistically significant inverse association between the extent of apoptosis and bcl-2 expression suggests that bcl-2 is one of the major regulators of apoptosis in pre-invasive and invasive breast tumours. However, since the material concerning some groups of breast lesions is rather small, the results can only be considered as preliminary and further studies are needed to substantiate the findings shown here.

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References


Apoptosis during Breast Carcinoma Progression

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ABSTRACT
The purpose of this study was to investigate apoptosis, proliferation, and the expression of apoptosis-influencing proteins bel-2 and bax and estrogen and progesterone receptors during breast carcinoma progression. The material consisted of 53 paired breast carcinoma samples representing primary and recurrent tumors and 24 control samples. The recurrent sample was located either in the breast scar tissue or at a distant metastatic site. Apoptosis was detected both morphologically and by 3' end labeling of fragmented DNA. Cell proliferation was evaluated immunohistochemically by the MIB index. The expressions of bel-2, bax, and estrogen and progesterone receptors were studied immunohistochemically. There was a significant increase in the extent of apoptosis and proliferation in recurrent tumors compared to the primary lesions (P = 0.015 and P = 0.038, respectively). In primary tumors with an apoptotic index of >0.50%, the survival of the patients was significantly shorter (P = 0.015). In cases with a significant increase in apoptosis or proliferation in the recurrent tumor, the survival of the patients was significantly shorter (P = 0.009 and P = 0.003, respectively). Of the variables analyzed, bel-2 expression and a positive estrogen receptor status were significantly associated with a low extent of apoptosis (P = 0.010 and P = 0.042, respectively). Their changes were parallel to the changes in apoptosis during tumor progression, although the associations did not reach statistical significance. The results show that increased apoptosis is associated with a worse prognosis in breast carcinoma. A significant increase in apoptosis in recurrent breast carcinoma lesions predicts a worse clinical outcome.

INTRODUCTION
Apoptosis is an actively regulated cellular process that leads to the destruction of individual cells (1–4). It can be triggered by several stimuli, such as radiation, drugs, and toxins, or by deprivation of hormones or growth factors (5, 6). Morphologically, apoptotic cells are characterized by nuclear shrinkage and pyknosis, eventually leading to nuclear fragmentation and phagocytosis of the apoptotic cells (3, 7). Biochemically, the end result of an apoptotic process is DNA fragmentation by endonucleases to periodic 180-bp fragments, which can be detected in DNA electrophoresis as a typical ladder pattern (3). The DNA fragmentation and nuclear destruction are regulated by caspases, which are proteolytic enzymes capable of cleaving amino acids at aspartic acid-asparagine residues (8, 9).

The importance of apoptosis in tissue homeostasis is reflected by the fact that it is influenced by several cancer genes, such as p53 and Rb tumor suppressor genes and c-myc (4, 5). An important group of genes influencing apoptosis is the bel-2 family (1–7). The proteins encoded by these genes can either promote or inhibit apoptosis (1–7). bel-2, for instance, is able to inhibit apoptosis (10). Translocation of the bel-2 gene to an active promoter site has been shown to be an important genetic change in the development of follicular lymphomas (11, 12). Other members of the bel-2-related group include bax, bad, bel-xL, bel-xS, mcl-1, and so on (1–5). These proteins may either homodimerize or form heterodimers with each other (1–5). The dimerization is important for the apoptosis regulatory function of these proteins (1–5). An important regulatory factor for apoptotic regulation in many cells is the balance between the concentrations of bax and bel-2 proteins. In cases in which bax is in excess, bax-bax homodimers, which lead to a promotion of apoptosis, predominate (4, 5). bel-2, however, may heterodimerize with bax, and its increased concentration leads to formation of bel-2-bax heterodimers, which favor abrogation of apoptosis (4, 5). Other members of the bel-2 group may also heterodimerize with bax (4, 5). bel-xL, for instance, by forming heterodimers with bax, is also able to inhibit apoptosis (5). bad, on the other hand, binds bel-2 and bel-xL, leading to an excess of bax homodimers and a promotion of apoptosis (5). Many members of the bel-2 family are resistant proteins of the mitochondrial membrane, and they have been shown to influence the mitochondrial membrane potential or to promote or inhibit the release of caspase-activating substances such as cytochrome c or apoptosis-inducing factor from mitochondria (13, 14). In line with their antiapoptotic functions, bel-2 and bel-xL inhibit the release of cytochrome c or apoptosis-inducing factor from mitochondria, whereas the proapoptotic bax promotes it (13, 14).

Apoptosis is often increased in malignant tumors (15, 16). In breast carcinoma, increased apoptosis is associated with a negative estrogen or progesterone receptor status, a low degree of differentiation, tumor aneuploidy, and a decreased bel-2 expression (17). Bel-2 expression is present in ~70% of breast carcinomas (18, 19). Strong bel-2 expression is associated with a positive estrogen or progesterone receptor status (20, 21). Its expression is related to a favorable prognosis (19, 20) but is not an independent prognostic factor (19). In node-positive breast carcinomas, however, bel-2 expression is independently related to a better prognosis (18, 20).
The aim of this study was to investigate apoptosis in a set of breast carcinomas from which samples of tumors could be obtained from at least two temporally different occasions. As a control group, we included 12 paired cases, in which samples were obtained from two histologically different tumors from different breasts of the same patients. The sections were also studied immunohistochemically for cell proliferation using the MIB-1 antibody and for the expression of bcl-2, bax, and estrogen and progesterone receptors to see whether changes in apoptosis could be ascribed to putative changes in their expression. The results were correlated with clinical data, such as survival of the patients.

**MATERIALS AND METHODS**

**Materials.** Fifty-three samples of breast carcinoma consisting of the primary and recurrent lesion and a control group of 12 cases representing two histologically different carcinomas from different breasts of the same patients were collected from the files of the Department of Pathology, University of Oulu, between the years 1979 and 1996. The material with the primary and recurrent samples consisted of 44 ductal carcinomas, 8 lobular carcinomas, and 1 mucinous carcinoma. In the control group there were 21 ductal carcinomas, 2 lobular carcinomas, and 1 apocrine carcinoma. The diagnosis was based on the WHO classification of breast tumors (22). The grades of the ductal carcinomas were evaluated according to Bloom and Richardson (23). All but seven tumors represented cases with nodal axillary metastases at the primary occasion. The total follow-up time between the primary and recurrent tumor in the paired sample group was 148.7 years (mean = 2.8 years, range = 0.3–10.9 years). In all but three cases, a full mastectomy was performed as an initial treatment. Forty patients received radiation therapy, 25 received antistrogen (tamoxifen), and 11 received cytostatic therapy during the follow-up.

**3' End Labeling of DNA in Apoptotic Cells.** To detect apoptotic cells, in situ labeling of the 3’ ends of the DNA fragments generated by apoptosis-associated endonucleases was used. The 3’ end labeling of DNA was performed using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD), as described previously (15, 16). A positive control consisted of a lymph node with follicular hyperplasia.

Cells were defined as apoptotic if their whole nuclear area labeled positively. Apoptotic bodies were defined as small positively labeled globular bodies in the cytoplasm of the tumor cells that could be found either singly or in groups. To estimate the apoptotic index, the numbers of apoptotic cells and bodies were counted in 10 HPFs with a total area of ~1.2 mm² (10 HPFs, ×40 objective, diameter of the field = 400 μm) and divided by the total number of tumor cells in this area. Cells in the vicinity of necrotic areas were not assessed.

In addition to the 3’ end labeling method, we also performed apoptosis assessment by light microscopy based on the morphological criteria of apoptosis (3). The morphological apoptosis was assessed from the same tumor samples, and the estimation of the apoptotic index was performed in a manner similar to that of the 3’ end labeling method.

**Immunohistochemical Staining.** Five-μm sections were cut from the specimens, dehydrated in xylene, and rehydrated in graded alcohol. The endogenous peroxidase was consumed by immersing the sections in 0.1% hydrogen peroxide in absolute methanol for 20 min. Nonspecific binding was blocked by incubating the slides in 20% FCS in PBS for 20 min.

For MIB-1 staining, a dilution of 1:25 was used for the primary monoclonal antibody (Immunotech, Marseilles, France). This was followed by a secondary biotinylated rabbit antimonuse antibody (dilution = 1:300; Dakopatts, Copenhagen, Denmark) and the avidin-biotin-peroxidase complex (Dakopatts).

A monoclonal antibody (clone 124) against bcl-2 oncoprotein was obtained from DAKO (Glostrup, Denmark). A polyclonal antibody to bax was obtained from PharMingen (San Diego, CA). Before application of the primary antibodies, the sections were heated in a microwave oven in 10 mm citric acid monohydrate (pH 6.0) for 5 min. After a 30-min incubation with the primary antibody (dilution = 1:50 for bcl-2 and 1:100 for bax), a biotinylated secondary antimonuse or antirabbit antibody (both from Dakopatts) was applied (dilution 1:300) followed by the avidin-biotin-peroxidase complex (Dakopatts).

For all of the immunostains, the color was developed by diaminobenzidine, after which the sections were lightly counterstained with hematoxylin and mounted with Buitkell (Kindler, Freiburg, Germany). Negative control stainings were carried out by substituting nonimmune mouse or rabbit serum for the primary antibodies. A positive control consisted of a lymph node with follicular hyperplasia.

The proliferative activity, expressed as the MIB index, was determined as the number of MIB-positive cells divided by the sum of all tumor cells in 10 HPF areas studied. The intensity of the bcl-2 and bax immunostaining was evaluated by dividing the cytoplasmic staining reaction in four score groups: 1 = weak cytoplasmic staining intensity; 2 = moderate cytoplasmic staining intensity; 3 = strong cytoplasmic staining intensity; and 4 = very strong cytoplasmic staining intensity. The immunostaining was quantified as follows: 0 = no positive immunostaining; 1 = <25% of tumor cells showing cytoplasmic positivity; 2 = 25–50% of tumor cells showing cytoplasmic positivity; 3 = 50–75% of tumor cells showing cytoplasmic positivity; and 4 = >75% of tumor cells showing cytoplasmic positivity. A combined score for bcl-2 and bax immunostaining, based on both qualitative and quantitative analysis, was obtained by adding the qualitative and quantitative scores; these sums were then divided in three main groups: score = 0, no immunoreactivity; score = 1–4, weak immunoreactivity; and score = 5–8, strong immunoreactivity.

For estrogen and progesterone receptor staining, the slides were rehydrated and then microwaved in EDTA buffer first 3 min at 99°C and then 27 min in 85°C. The endogenous peroxidase was blocked with 0.1% hydrogen peroxide in absolute methanol for 20 min. Nonspecific staining was blocked by incubating the slides in normal FCS for 20 min, followed by the primary antibody and the avidin-biotin-peroxidase complex. The slides were counterstained with methyl green, and the
estrogen and progesterone receptor status was determined as described previously (24).

Statistical Analysis. SPSS for Windows (Chicago, IL) was used for statistical analysis. The significance of the associations were determined using the χ² test, Fisher's exact probability test, correlation analysis, and two-tailed t test. Univariate and multivariate analyses of survival data were undertaken by using survival curves and applying the Kaplan-Meier method with log-rank analysis and the Cox regression model. Probabilities of P ≤ 0.05 were regarded as statistically significant.

RESULTS

Apoptotic and MIB Indices in Primary and Recurrent Breast Carcinomas. The average apoptotic index in all samples was 0.97 ± 1.27% (mean ± SD; range = 0.00–5.85%), and the MIB index was 9.26 ± 10.92% (range = 0.09–70.68%). There was a positive correlation between the apoptotic and MIB index (r = 0.3997, P < 0.001).

The average apoptotic index in the primary carcinoma lesions was 0.75 ± 1.02% (range = 0.00–5.85%), whereas in the recurrent tumors, it was 1.36 ± 1.42% (range = 0.00–5.37%; P = 0.015). In the 12 control cases from patients with two histologically different breast tumors, the mean apoptotic index did not show a statistically significant difference between the tumors (0.35 ± 0.56% and 0.18 ± 0.13%, respectively, P = 0.41).

The average MIB index in the primary breast carcinomas was 7.17 ± 7.55% (range = 0.14–51.86%), and in the recurrent carcinomas, it was 11.41 ± 13.27% (range = 0.32–70.45%; P = 0.038). In the control cases, no significant difference was observed (5.76 ± 5.74% and 5.74 ± 5.25%, respectively; P = 0.85).

Of the recurrent tumors, 16 were local recurrences, 23 were lymph node metastases of axillary or other sites, and the rest were metastases in other locations (skin, peritoneum, gastrointestinal tract, and liver). The apoptotic index was highest in recurrences in lymph nodes (1.35 ± 1.45%, P = 0.07 compared to the primary tumor) and in local recurrences in the breast tissue scar (1.31 ± 1.59%; P = 0.10), but it was somewhat lower in recurrences at other sites (0.95 ± 1.33%; P = 0.51; Fig. 1).

The MIB index was highest in local recurrences (18.72 ± 8.37%), as compared to the primary tumor (P = 0.001; Fig. 2). The MIB index in lymph nodes or other sites did not significantly differ from the primary tumor (P = 0.50 and 0.26, respectively; Fig. 2).

Patients with a primary tumor showing an apoptotic index of >0.50% had a significantly shorter survival (P = 0.015, log-rank; Fig. 3). Although patients with a high MIB index (>7.18%) in the primary tumor had a slightly worse prognosis, the difference was not statistically significant (P = 0.50, log-rank). Patients showing a >30% increase in apoptosis or a >2.00% proliferation per year had a significantly shorter survival than other cases (P = 0.009 and 0.0027, respectively; Figs. 4 and 5).

bcl-2, bax, and Estrogen and Progesterone Receptors in Primary and Recurrent Breast Tumors. A decrease in bcl-2 and bax immunoreactivity in recurrent versus primary tumors sample could be seen in 4 and 3 cases, respectively, and an increase was seen in 10 and 11 cases. A loss of a positive estrogen or progesterone receptor status could be seen in 6 and 2 cases, respectively. In cases with an increased bcl-2 immunoreactivity in the recurrent tumor, there was an average increase of only 0.15 ± 0.46% in the apoptotic index while in cases with no change or a decrease it was 0.71 ± 1.62% (P = 0.10). An increase in bax expression was not significantly associated with changes in apoptosis in the recurrent tumor (0.62 versus 0.56%, P = 0.57). In cases with a loss of positive estrogen or progesterone receptor status, there was an average increase of 1.39 ± 2.59% in apoptosis, whereas in the other cases, it was only 0.41 ± 1.29% (P = 0.17). Cases with a loss in the positive estrogen or progesterone status showed a significant increase in proliferation (P = 0.029). Changes in bcl-2 or bax expression did not significantly affect proliferation (data not shown).

Associations between bcl-1 and bax expression and estrogen and progesterone receptor status in primary and recurrent tumors are given in Table 1. There was no significant associa-
Fig. 3. Apoptosis and survival in primary breast carcinoma. The cumulative survival of patients with primary tumors showing an apoptotic index of >0.50% is significantly shorter than that of the other patients (P = 0.015, log-rank); —, apoptosis > 0.50%; — —, apoptosis ≤ 0.50%.

Fig. 4. Increased apoptosis rate and survival. In cases in which the apoptotic index in the recurrent tumor had increased >0.50% per year compared to the primary tumor, the survival of the patient was significantly shorter (P = 0.009, log-rank). Increased apoptosis: — —, change > 0.50%; — — —, change ≤ 0.50%.

Fig. 5. Increase proliferation rate and survival. In cases in which the MIB index in the recurrent tumor had increased >2.00% per year compared to the primary tumor, the survival of the patient was significantly shorter (P = 0.0027, log-rank). Proliferation change: — —, change > 2.00%; — — —, change ≤ 2.00%.

P = 0.01, respectively, log-rank). The bax immunoreactivity, on the other hand, did not affect the survival (P = 0.86, log-rank).

Other Variables. Anti-oestrogen and cytostatic treatment did not significantly affect the rate of apoptosis or tumor cell proliferation in the recurrent tumors (P = 0.46 and P = 0.97 (anti-oestrogen treatment) and P = 0.37 and P = 0.13 (cytostatic treatment)). Of 25 cases receiving anti-oestrogen therapy, 11 showed an apoptotic index of <0.50%. The corresponding figures for cases receiving radiation therapy and cytostatic treatment during the follow-up were 18 of 40 and 5 of 11, respectively. By Cox multivariate regression model, enhanced apoptosis showed a 2.0-fold risk for a shortened survival (P = 0.016, 95% confidence interval = 1.14–3.49) and appeared to be an independent prognostic variable, whereas synergistic treatment (P = 0.10, tamoxifen (P = 0.72), and radiation therapy (P = 0.99) did not show such an association.

Comparison of the Morphological Apoptotic Index with the 3' End Labeling Method. The average morphological apoptotic index was 0.87 ± 1.01% (range = 0.01–4.95%), giving a slightly lower index than 3' end labeling. There was a statistically significant positive correlation between the morphological apoptotic index and the 3' end labeling method (r = 0.494, P < 0.001). The morphological apoptotic index was clearly lower in primary (0.69 ± 0.62%, range = 0.01–2.98%) than in recurrent (1.14 ± 1.30%, range = 0.01–4.96%) tumors (P = 0.04). With the other parameters, similar results were obtained. There was, for instance, a statistically significant association between a high morphology apoptotic index and a low bcl-2 expression (P = 0.005) or between a high morphological apoptotic index and a low estrogen receptor status (P = 0.016). There was also a statistically significant difference in the survival of patients with tumors showing an increase in apoptosis in the recurrent tumor compared with other tumors (P = 0.046, log-rank).

DISCUSSION

In this study, we investigated apoptosis and cell proliferation in breast carcinomas by analyzing specimens from the
Table 1  bcl-2, bax, and estrogen and progesterone receptor expression in primary and recurrent tumors

<table>
<thead>
<tr>
<th>Primary tumor</th>
<th></th>
<th>PR*</th>
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</tr>
</thead>
<tbody>
<tr>
<td>bcl-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak expression</td>
<td>21</td>
<td>6</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td>Strong expression</td>
<td>10</td>
<td>13</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>P*</td>
<td>0.014</td>
<td>0.017</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bax</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak expression</td>
<td>21</td>
<td>13</td>
<td>25</td>
<td>9</td>
</tr>
<tr>
<td>Strong expression</td>
<td>8</td>
<td>7</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>P*</td>
<td>0.808</td>
<td>0.799</td>
<td></td>
<td></td>
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<tr>
<td>Recurrent tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bcl-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak expression</td>
<td>18</td>
<td>3</td>
<td>20</td>
<td>2</td>
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<tr>
<td>Strong expression</td>
<td>17</td>
<td>12</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>P*</td>
<td>0.038</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bax</td>
<td></td>
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<tr>
<td>Weak expression</td>
<td>19</td>
<td>6</td>
<td>18</td>
<td>7</td>
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<tr>
<td>Strong expression</td>
<td>14</td>
<td>10</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>P*</td>
<td>0.155</td>
<td>0.273</td>
<td></td>
<td></td>
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</tbody>
</table>

* ER, estrogen receptor; PR, progesterone receptor.
* According to Fisher’s exact test.

The results show that there is a significant increase in apoptosis and proliferation in recurrent tumors compared to the primary tumors. According to the results, the increase in both apoptosis and cell proliferation also influences patient prognosis. In cases in which the increase in apoptosis or proliferation in the recurrent tumor was high, the patients had a shorter survival. This probably depends on alterations in the expression of genes influencing apoptosis or proliferation during tumor progression, which, in turn, also influences the prognosis of the patients.

To study such alterations, we also analyzed changes in the expression of apoptotic genes including bcl-2 and bax proteins and estrogen and progesterone receptors during tumor progression. Genes of the bcl-2 group are known to affect apoptosis, and bcl-2 and bax expression, especially, have been shown to be important in breast carcinoma (18–21, 25). In line with this in all tumor samples, we found a significant inverse association between bcl-2 expression and apoptosis. bcl-2 was also associated with a positive estrogen and progesterone receptor status, both of which also were inversely associated with the extent of apoptosis. The study, thus, supports the previously reported hypothesis that a positive estrogen or progesterone receptor status might up-regulate bcl-2 expression, which could lead to a decrease in apoptosis (26). With bax, no such associations could be found, suggesting that, in breast tumors, bcl-2 expression is more crucial in determining the extent of apoptosis.

When bcl-2 and bax expression in primary and recurrent tumors were compared, a change in their expression was seen in about a half of the cases, and a loss of a positive estrogen or progesterone receptor status was seen in 15%. Cases with an increased bcl-2 expression in the recurrent tumor had a lower level of apoptosis, suggesting that up-regulation of bcl-2 expression in recurrent tumors might lead to a lower level of apoptosis in them. On the other hand, tumors with a loss of a positive estrogen or progesterone receptor status showed an increase in apoptosis and proliferation in the recurrent tumors. The data, thus, show that changes in bcl-2 or the estrogen and progesterone receptor status are parallel to the changes seen in apoptosis or proliferation during breast carcinoma progression.

Here, we also tested whether the location of the recurrence might influence apoptosis or proliferation. Theoretically, this might be possible through interaction of adhesion molecules on the tumor cells with the local matrix. In keeping with this, it has been shown that loss of cellular adhesion by blocking integrin binding to the extracellular matrix by specific antibodies may lead to apoptosis of the cells (27, 28). To study site-specific differences in apoptosis and proliferation, we compared the two phenomena separately in recurrences of the breast scar tissue, lymph nodes, and other sites. Although the extent of apoptosis was higher in all locations compared to the primary tumor lesion, no significant difference was observed in apoptosis between the different recurrent tumor sites, suggesting that the local milieu does not play any significant role in determining the extent of apoptosis in them. On the other hand, the MIB index was significantly higher in recurrent tumors in the breast scar tissue than in other sites, suggesting that cell populations with a higher proliferative activity have a selective advantage over cells with a lower proliferative capacity in this site. Rather than being due to adhesion molecules, this phenomenon may reflect a selective difference between cell populations in local recurrences compared to recurrences in metastatic sites; in cell populations leading to local recurrences, genetic changes favoring an increased cell proliferation could play a more significant role compared to cell populations with a metastatic phenotype.

In our study, apoptosis in the primary breast carcinoma lesions was also associated with a shorter survival of the patients. This is in line with a previous study of Lipponen et al. (17), who used only morphology in the assessment of apoptosis. Increased apoptosis is also associated with a shortened survival in other epithelial tumors, such as non-small cell lung carcinomas (15).

In previous reports it was suggested that the in situ 3’ end labeling would be unreliable in detecting apoptosis because factors like fixation, DNA damage, or necrosis might influence the results (29, 30). Because of this, we also assessed apoptosis morphologically in H&E-stained slides from the same tumor sections. The results show a strong correlation between morphological apoptosis and the apoptotic index obtained with the 3’ end labeling method. This is in line with the results obtained by other authors (31, 32). Moreover, the morphological apoptotic index showed similar associations as with the 3’ end labeling method. Because the apoptotic index with 3’ end labeling method was slightly higher, it might even be more sensitive in detecting apoptotic cells.

In conclusion, the results show that apoptosis and proliferation are increased during breast carcinoma progression. An increased apoptosis in primary breast carcinoma is associated with a shorter survival of the patients. Furthermore, a significant increase in apoptosis or proliferation in recurrent tumor compared to the primary lesion also predicts a poorer prognosis.

REFERENCES
Expression of caspases 3, 6 and 8 is increased in parallel with apoptosis and histological aggressiveness of the breast lesion

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Summary The aim of this investigation was to study the expression of caspases 3, 6 and 8 and their association to apoptosis in preneoplastic and neoplastic lesions of the breast. The material consisted of nine benign breast epithelial hyperplasias, 15 atypical hyperplasias, 74 in situ and 82 invasive carcinomas. The extent of apoptosis was assessed by the TUNEL method and caspase 3, 6 and 8 expression by immunohistochemistry with specific antibodies. Increased caspase 3 immunopositivity, as compared to staining of normal breast ductal epithelium, was seen in 22% of benign epithelial hyperplasias, 25% of atypical hyperplasias, 59% of in situ carcinomas and 90% of invasive carcinomas. The corresponding percentages for caspase 6 and 8 were 11%, 25%, 60%, 87% and 22%, 57%, 84%, 83% respectively. In high-grade in situ lesions there were significantly more cases with strong caspase 3, 6 and 8 immunoreactivity than in low- and intermediate-grade lesions ($P = 0.0045$, $P = 0.049$ and $P = 0.0001$ respectively). In invasive carcinomas, however, no association between a high tumour grade and caspase 3, 6 or 8 expression was found ($P = 0.27$, $P = 0.26$ and $P = 0.69$ respectively). The mean apoptotic index was $0.14 \pm 0.14$% in benign epithelial hyperplasias, $0.17 \pm 0.12$% in atypical hyperplasias, $0.81 \pm 0.88$% in situ carcinomas and $0.94 \pm 1.21$% in invasive carcinomas. In all cases strong caspase 3, 6 and 8 positivity was significantly associated with the extent of apoptosis ($P < 0.001$, $P = 0.015$ and $P = 0.050$ respectively). The results show that synthesis of caspases 3, 6 and 8 is up-regulated in neoplastic breast epithelial cells in parallel to the increase in the apoptotic index and progression of the breast lesions. © 1999 Cancer Research Campaign

Keywords: apoptosis; caspase; breast; carcinoma

Caspases are molecules involved in the terminal execution of apoptosis (Thornberry et al, 1994; Alnemri et al, 1996; Barge et al, 1997; Harvey et al, 1997; Thornberry and Lazebnik, 1998). They are activated in a cascade-like fashion and are able to cleave substrate proteins at a consensus sequence following aspartic acid residues which may, however, be different for different caspases (Pestel et al, 1996; Palaeiro et al, 1997; Thornberry and Lazebnik, 1998). Their substrates include DNA repair enzymes, such as poly(ADP-ribose)polymerase, several structural proteins of the cells, such as nuclear lamin, fodrin, B-catenin and cytokeratin 18, oncoproteins such as mdm2 and tumour suppressor gene products such as retinoblastoma protein (Pestel et al, 1996; Rao et al, 1996; Brancolini et al, 1997; Caslin et al, 1997; Chen et al, 1997; Tan et al, 1997). Caspases are also able to activate DNAase and are thus required for the typical DNA fragmentation found in apoptosis (Esori et al, 1998; Janicke et al, 1998). They are resident proteins of the cytosol and when activated, the amino-terminal fragment of the molecule is removed and the rest of the molecule is cleaved into 10- and 20-kDa fragments, which form an active αβ tetrameric structure (Thornberry et al, 1994; Martins et al, 1997; Thornberry and Lazebnik, 1998).

There are at least 13 mammalian caspases known so far (Thornberry and Lazebnik, 1998). They can be divided in subgroups according to their phylogenetic development, structure or their order in the caspase activation cascade (Alnemri et al, 1996; Barge et al, 1997; Harvey et al, 1997). Caspases can be activated by mitochondrial substances such as cytochrome c or apoptosis-inducing factor (AIF) the release of which is induced by changes in the mitochondrial membrane permeability or electric potential (Liu et al, 1996; Kluck et al, 1997; Manon et al, 1997; Yang et al, 1997). Release of cytochrome c, for instance, first leads to activation of caspase 9 which then activates caspase 3 (Nijhawan et al, 1997). The changes in the mitochondrial membranes is influenced by members of the bcl-2 family proteins (Kluck et al, 1997; Manon et al, 1997; Yang et al, 1997). These proteins modulate the apoptotic response and they can be either pro- or antiapoptotic (Yang and Korsmeyer, 1996; Kroemer, 1997). Their structure is reminiscent of membrane pore forming proteins, such as cholera toxins, and bcl-XL and bcl-2 have been shown to inhibit release of cytochrome c or AIF from mitochondria while bax has been shown to promote it (Kluck et al, 1997; Manon et al, 1997; Mimm et al, 1997; Yang et al, 1997).

The caspase cascade can also be activated directly through activation of the tumour necrosis factor (TNF) receptors such as APO-1/FAS/CD95. The APO-1/FAS/CD95 receptor is stimulated by binding of the FAS ligand (Fraser and Evan, 1996; Nagata, 1997). Upon attachment with the Fas ligand the APO-1/FAS/CD95 receptor oligomerizes, and FADD/MORT1 and FLICE (procaspase 8) become associated with it, forming the so-called death-inducing signal complex (DISC) (Muzio et al, 1996; Nagata, 1997). This is followed by activation of procaspase 8, which leads to the activation of other downstream caspases and to an apoptotic demise of the cell (Muzio et al, 1996; Nagata, 1997).
III

Table 1  The extent of apoptosis and caspase 3, 6 and 8 immunoreactivity in hyperplasias, atypical hyperplasias, in-situ and invasive carcinomas of the breast

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Apoptosis %</th>
<th>Caspase 3</th>
<th>Caspase 6</th>
<th>Caspase 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>0.14 ± 0.14</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Atypical hyperplasia</td>
<td>0.17 ± 0.12</td>
<td>9</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>In-situ carcinoma</td>
<td>0.01 ± 0.08</td>
<td>27</td>
<td>21</td>
<td>16</td>
</tr>
<tr>
<td>Comedo</td>
<td>1.09 ± 1.28</td>
<td>5</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Solid</td>
<td>0.79 ± 0.81</td>
<td>3</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Cribriform</td>
<td>0.52 ± 0.78</td>
<td>10</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Papillary</td>
<td>0.17 ± 0.13</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Lobular</td>
<td>0.20 ± 0.10</td>
<td>4</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>0.94 ± 1.21</td>
<td>7</td>
<td>14</td>
<td>49</td>
</tr>
<tr>
<td>Ductal</td>
<td>1.00 ± 1.26</td>
<td>4</td>
<td>13</td>
<td>44</td>
</tr>
<tr>
<td>Lobular</td>
<td>0.54 ± 0.80</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Muchous</td>
<td>0.47 ± 0.79</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

+ = negative or weakly positive, ++ = moderately positive, +++ = strongly positive.

Several studies have shown that apoptosis is increased in malignant tumors (reviewed in Soini et al. 1998). In breast carcinomas, high-grade lesions display a higher apoptotic index than low-grade lesions (Lippinen et al. 1994). Apoptosis in breast carcinoma is also associated with other parameters, such as proliferation, tumour ploidy and survival and it is inversely associated with a positive oestrogen or progesterone receptor status and bcl-2 expression (Lippinen et al. 1994; Mustonen et al. 1997). The anti-apoptotic effect of a positive oestrogen receptor status is mediated through stimulation of bcl-2 mRNA synthesis by oestrogen (Wang and Phang, 1995). Decreased expression of pro-apoptotic bax is associated with a poor prognosis of the breast carcinoma patients due to development of resistance to chemotherapeutic agents (Wang and Phang, 1995).

The immunohistochemical expression and distribution of different caspases and their relation to apoptosis in breast carcinomas and preneoplastic lesions has not previously been studied. In this study we evaluated the immunohistochemical distribution of caspases 3, 6 and 8 in preneoplastic and neoplastic lesions of the breast and their relation to the apoptotic index, as determined by the TUNEL method. These three caspases were chosen because of their known central roles in apoptosis (Muzio et al. 1996; Faleiro et al. 1997).

MATERIALS AND METHODS

Samples

One hundred and eighty breast lesions consisting of 82 invasive carcinomas, 74 in situ carcinomas, 15 atypical hyperplasias and nine benign epithelial hyperplasias were collected from the files of the Department of Pathology, Oulu University Hospital. All material was fixed in 10% neutral formalin and embedded in paraffin. The invasive carcinomas consisted of 71 ductal, seven lobular, two mucinous, one apocrine and one tubular carcinoma. The in situ carcinomas consisted of 26 cribriform, 18 comedo, 11 solid, seven papillary and 12 lobular in situ lesions. The 15 atypical hyperplasias consisted of 13 ductal and two lobular hyperplasias. The diagnosis was based on the AFIP classification of breast tumours (Rosen and Oberman, 1992). The grades of the ductal carcinomas were evaluated according to the criteria of Bloom and Richardson, and the grades of the in situ lesions according to the criteria of Holland et al (Holland et al. 1990; Elston and Ellis, 1991). The atypical hyperplasias were defined according to Tavassoli (1992). First, the apoptotic index was determined from all these tumours. Caspase 3, 6 and 8 immunohistochemistry was performed from tumour blocks where representative areas of the breast lesions were still available after the apoptotic labelling had been performed. The number of these cases is shown in Table 1. The mean follow-up was 5.7 ± 2.9 years. There were 20 stage I, 21 stage II, 21 stage III and 11 stage IV tumours. Anti-oestrogenic treatment was given to 40 patients, 35 patients received cytostatic treatment and 30 radiation therapy in some stage of the disease.

Immunohistochemical stainings

Polyclonal rabbit anti-human caspase-3 antibody was purchased from Pharmingen (San Diego, CA, USA). According to the manufacturer, the antibody recognizes both the unprocessed 32 kDa pro-caspase-3 molecule and the fragmented larger active 17 kDa unit. Polyclonal goat anti-human antibodies to caspase 6 (mch2) and caspase 8 (mch5) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). According to the manufacturer, the mch2 antibody recognizes amino acids 157–176 and mch5 amino acids 354–373 of the carboxyl terminal part of the protein, which both belong to the processed p20 fragment of the protein. The two antibodies thus recognize both the unprocessed and the cleaved or activated form of both caspases.

Before application of the primary antibodies, the sections were heated in a microwave oven in 10 mm citric acid monohydrate, pH 6.0, for 5–10 min. After a 60-min incubation with the primary antibody (dilution 1:500 for anti-caspase 3, 1:100 for anti-caspase 6 and 8), a biotinylated secondary anti-rabbit or anti-goat antibody (all three from Dakopatts, Copenhagen, Denmark) was applied (dilution 1:200–300) followed by the avidin–biotin–peroxidase complex (Dakopatts).

For all immunostainings, the colour was developed by diamobenzidine, whereas after the sections were lightly counterstained with methyl green and mounted with Eukitt (Kindler, Freiburg, Germany).
Negative control stainings were carried out by substituting non-immune goat or rabbit serum for the primary antibodies. As a positive control for the immunostainings, a lymph node with follicular hyperplasia was used.

The intensity of the immunostainings with all the antibodies was evaluated by dividing the staining reaction in four groups:

- 1 = weak cytoplasmic staining intensity
- 2 = moderate cytoplasmic staining intensity
- 3 = strong cytoplasmic staining intensity
- 4 = very strong cytoplasmic staining intensity.

The quantity of the immunostaining was evaluated as follows:

- 0 = No positive immunostaining
- 1 = < 25% of tumor cells showing cytoplasmic positivity
- 2 = 25–50% of tumor cells showing cytoplasmic positivity
- 3 = 50–75% of tumor cells showing cytoplasmic positivity
- 4 = > 75% of tumor cells showing cytoplasmic positivity.

A combined score for the immunostaining, based on both qualitative and quantitative immunostaining, was composed by adding both the qualitative and quantitative score which was then divided into three groups:

- + = no or weak immunostaining; score 0–2
- ++ = moderate immunostaining; score 3–5
- +++ = strong immunostaining; score 6–8.

For oestrogen and progesterone receptor staining, the slides were rehydrated and then microwaved in EDTA buffer first for 3 min in 99°C and then for 27 min in 85°C. The endogenous peroxidase was blocked with 0.1% hydrogen peroxide in absolute methanol for 20 min. Non-specific staining was blocked by incubating the slides in normal fetal calf serum for 20 min, followed by the primary antibody and the avidin–biotin–peroxidase complex. The slides were counterstained with methyl green, and the oestrogen and progesterone receptor status was counted as previously described (Helin et al, 1989).

### 3′-end labelling of DNA in apoptotic cells

In order to detect apoptotic cells, in situ labelling of the 3′-ends of the DNA fragments generated by apoptosis-associated endonucleases was performed using the ApoTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD, USA) as previously described (Törninen et al, 1995; Soini et al, 1996). The sections, after being dehydrated in xylose and rehydrated in ethanol, were incubated with 20 µg ml⁻¹ Proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) at room temperature for 15 min. The endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in PBS, pH 7.2. The slides were then treated with terminal deoxynucleotidyl transferase enzyme and digoxigenin-labelled nucleotides after which anti-digoxigenin-peroxidase solution was applied on the slides. The colour was developed with diaminobenzidine after which the slides were lightly counterstained with haematoxylin. For control purposes we used tissue sections from hyperplastic lymph nodes showing an increased number of apoptotic B-cells within germinal centres and a low number of apoptotic T-cells in the interfollicular areas.

### Assessment of the apoptotic index

Cells were defined as apoptotic if the whole nuclear area of the cell labelled positively. Apoptotic bodies were defined as small positively labelled globular bodies in the cytoplasm of the tumour cells which could be found either singly or in groups. To estimate the apoptotic index (the percentage of apoptotic events in a given area), apoptotic cells and bodies were counted in 10 high-power fields (HPFs) and this figure was divided by the number of tumour cells in the same HPFs.

In order to test the reproducibility of estimation of apoptosis by the TUNEL method we also performed apoptosis assessment by light microscopy in invasive breast carcinoma samples. The assessment was based on the morphological criteria of apoptosis described previously (Kerr et al, 1994). The morphological apoptosis was assessed from the same tumour samples and the estimation of the apoptotic index was performed in a similar manner as with the 3′-end labelling method.

### Statistical analysis

Comparisons between groups were made using the Mann–Whitney U-test. The significance of associations was determined using Fisher’s exact probability test and correlation analysis. Survival was analysed by applying the Kaplan–Meier method with log-rank analysis. Probability values ≤ 0.05 were considered significant.
RESULTS

Caspase 3, 6 and 8 expression in non-neoplastic tissues

Diffuse, weak (+) and inconsistent cytoplasmic positivity was seen for caspases 3, 6 and 8 in non-neoplastic ductal and lobular cells (Figure 1). The immunoreactivity was slightly stronger in benign epithelial lesions, like in areas with fibrocystic changes and it was also pronounced in areas with inflammation. Curiously, ducts with apocrine metaplasia frequently showed strong diffuse cytoplasmic staining for caspases, especially caspase 8.

Caspase 3, 6 and 8 expression in breast preneoplastic and neoplastic lesions

The results of the study are compiled in Table 1. Expression of caspases 3, 6 and 8 in breast preneoplastic and neoplastic cells was mainly diffuse, intracytoplasmic (Figures 2 and 3). Caspase 6 and 8, however, also expressed granular intracytoplasmic positivity the amount of which, however, varied from tumor to tumor (Figure 4). This reaction pattern was especially prominent in invasive carcinomas and many times gave an impression of being located in the apoptotic bodies. Granular caspase 6 or 8 positivity did not, however, associate with the apoptotic index (P = 0.19 and P = 0.98 respectively). In a few cases granular staining was also seen with caspase 3. Interestingly, three carcinoma cases showed also nuclear caspase 3 staining. Nuclear staining was not seen with caspases 6 and 8. Caspase 8, on the other hand, sometimes expressed membrane-associated staining.

There was an increase in the immunoreactivity of all three caspases in parallel with the histological progression of the breast lesion. Increased caspase 3 immunoreactivity (+++, ++++) as compared to benign ductal epithelium (+) was seen in 22% of benign epithelial hyperplasias, 25% of atypical hyperplasias, 58% of in situ carcinomas and 90% of invasive carcinomas. The corresponding percentages for caspase 6 were 11%, 25%, 60% and 87%, and for caspase 8 were 22%, 57%, 84% and 83%. There was a strong association between the expression of different caspases in atypical hyperplasias and carcinomas. Concomitant strong (+++), moderate (++), weak or negative (+) caspase 3 and 6 staining was seen in 79% of the cases (P = 0.00001). The corresponding percentages for caspase 3 and 8 and caspase 6 and 8 were 74% (P = 0.00057) and 71% (P = 0.01).

There were significantly more cases with increased (+++, ++++) caspase 3 immunoreactivity in invasive breast carcinomas than in in situ carcinomas (P = 0.00002), atypical hyperplasias (P = 0.00001) or benign epithelial lesions (P = 0.00004). Also, in-situ carcinomas had more cases with increased (+++, ++++) caspase 3 immunoreactivity than atypical hyperplasias (P = 0.037) or benign epithelial hyperplasias (P = 0.049). Similarly, invasive breast carcinomas had more cases showing increased (+++, ++++) caspase 6 positivity than in-situ carcinomas (P = 0.00024), atypical hyperplasias (P = 0.00002) or benign epithelial hyperplasias (P = 0.00001), and in-situ carcinomas showed more cases with increased caspase 6 immunoreactivity (+++, ++++) than atypical hyperplasias (P = 0.025) or benign epithelial hyperplasias (P = 0.00657). With caspase 8, however, invasive breast carcinomas did not show significantly more cases with increased (+++, ++++) positivity than in-situ carcinomas (P = 0.57) or atypical hyperplasias (P = 0.99), but there were more cases than in benign epithelial hyperplasias (P = 0.00035). Also, in-situ carcinomas did not have significantly more cases with increased (+++, ++++) caspase 8 positivity than atypical hyperplasias (P = 0.99), but the difference with benign epithelial hyperplasias was significant (P = 0.00032). Invasive breast carcinomas, however, showed significantly more cases with strong (+++, ++++) caspase 8 positivity than in-situ carcinomas (P = 0.02) or atypical hyperplasias (P = 0.0008), and in-situ carcinomas showed significantly more cases with strong
Figure 3  Caspase staining in breast invasive carcinomas. Strong staining for caspase 3 can be seen in invasive ductal (A) and for caspase 6 in invasive lobular (B) carcinoma

Figure 4  Granular caspase 6 staining in invasive breast carcinoma. The black dots in the figure correspond to positively stained fragments which are reminiscent of apoptotic cells and bodies (A). In this figure caspase 6 immunostaining in a lymphatic follicle from the control lymph node is shown. Apoptotic cells and fragments show a similar positivity as shown in the breast carcinoma (B)

(++) caspase 8-positive cases than atypical hyperplasias ($P = 0.02$).
Similar associations were found with caspase 3 and 6 ($P < 0.00001$, $P = 0.00008$, $P = 0.057$ for caspase 3 respectively, and $P = 0.006$, $P = 0.00002$ and $P = 0.12$ for caspase 6 respectively).

Low- and intermediate-grade in-situ lesions of the breast had significantly fewer cases with strong (++) caspase 3, 6 and 8 immunoreactivity than high-grade lesions ($P = 0.0045$, $P = 0.049$, $P = 0.0001$ respectively). In invasive carcinomas, however, a similar association between strong (++) caspase immunoreactivity and high-grade tumours was not found ($P = 0.27$, $P = 0.26$ and $P = 0.69$ respectively).

Apoptotic index in invasive and in-situ carcinomas and in atypical and benign epithelial hyperplasias

The apoptotic index in benign epithelial hyperplasias, atypical hyperplasias, in situ carcinomas and invasive carcinomas are shown in Table 1. There was a significantly lower apoptotic index in benign and atypical hyperplasias compared to in situ or invasive carcinomas ($P < 0.001$). Invasive carcinomas displayed a higher apoptotic index than atypical ($P < 0.012$) or benign epithelial hyperplasias ($P < 0.001$). Also, in situ carcinomas had a higher apoptotic index than atypical ($P = 0.004$) or benign epithelial hyperplasias ($P < 0.001$). No difference was, however, observed in the apoptotic index between atypical and benign epithelial hyperplasias ($P = 0.97$).

Low-grade in situ lesions showed a significantly lower extent of apoptosis ($0.23 \pm 0.20\%$) than intermediate- and high-grade lesions ($1.05 \pm 1.15\%$) ($P < 0.001$). Similarly, low- and intermediate-grade lesions showed a significantly lower extent of apoptosis than comedo-type lesions ($0.51 \pm 0.73\%$) than high-grade lesions ($1.04 \pm 1.25\%$) ($P = 0.012$). In situ lobular carcinomas showed a significantly lower extent of apoptosis than comedo-type lesions ($P < 0.001$).

The apoptotic index in grade I invasive tumours was lower ($0.75 \pm 0.71\%$) than in grade II ($0.95 \pm 1.55\%$) or grade III ($1.10 \pm 1.10\%$) lesions and lobular invasive carcinomas had a lower apoptotic index ($0.54 \pm 0.60\%$) than ductal carcinomas ($1.00 \pm 1.26\%$). There was, however, no significant difference
between invasive grade I and grade II-III or grade I-II and grade III tumours (P = 0.42 and P = 0.22 respectively) or between invasive ductal or lobular carcinomas (P = 0.97).

Association of caspases 3, 6 and 8 with apoptosis, oestrogen and progesterone receptor status and survival

In all cases, strong (+++) caspase 3 positivity was significantly associated with increased apoptosis (1.00 ± 1.27% for strong expression, 0.36 ± 0.56% for other cases, P = 0.007). A similar association was seen with caspase 6 (1.11 ± 1.21% vs 0.62 ± 0.99%, P = 0.012) and caspase 8 (1.06 ± 1.24% vs 0.53 ± 0.90%, P = 0.009). There was no significant association between strong (++++) caspase 3, 6 or 8 immunoreactivity and a positive oestrogen or progesterone receptor status in invasive carcinomas (P = 0.29, P = 0.53, P = 0.54; and P = 0.33, P = 0.45 and P = 0.08 respectively). In invasive carcinomas, no association with patient survival was observed with caspase 3, 6 or 8 (P = 0.77, P = 0.94 and P = 0.14 respectively).

Comparison of the TUNEL method with light microscopy

To study the reproducibility of the TUNEL method, we also evaluated the apoptotic index by morphological criteria in invasive breast carcinomas and compared the values with those obtained by TUNEL. The average apoptotic index in invasive carcinomas was 0.82 ± 0.97%. According to the results, there was a strong positive correlation between the extent of apoptosis obtained by TUNEL and light microscopic morphology (r = 0.5018, P < 0.001).

DISCUSSION

This study was undertaken to investigate the expression of caspases 3, 6 and 8 in benign and atypical hyperplasia, and in situ and invasive carcinomas of the breast. Also, the extent of apoptosis was studied and the results were correlated with the expression of the caspases in the lesions.

According to the results there was a gradual increase in the expression of caspases 3, 6 and 8 along with the progression of the breast lesion. Moreover, the expression of all these caspases was strongly associated with the rise in the apoptotic index. The results thus indicate increased synthetic activity of these proteins during neoplastic progression of breast epithelial cells. Immunohistochemistry, however, cannot discriminate between the active and inactive forms of the caspases, and only the active form is responsible for the effects of the caspases leading to apoptosis. AIF may, however, up-regulate the synthesis of caspases prior to activating them. Topoisomerase II inhibitor etoposide, for instance, up-regulates caspases 2 and 3 in neoplastic haematopoietic cell lines prior to induction of apoptosis, and up-regulation of caspase 2 mRNA has been shown in response to ischaemia-induced cell death (Harvey et al., 1997; Droni et al., 1998).

In our recent study, caspase 3, 6 and 8 immunoreactivity was found in 70-80% of pancreatic adenocarcinomas (Virkarvij et al., 1998). In lymphomas caspase 3 expression was increased in the large cell population of large- and mixed-cell follicular non-Hodgkin’s lymphomas and in Reed-Sternberg cells of Hodgkin’s disease (Chhanabhai et al., 1997; Knjewski et al., 1997). There may, however, be differences in the expression of caspases in histologically different tumours and tumours of different sites. In cell line studies, the expression of caspase mRNA has been shown to be heterogeneous (Droni et al., 1998). Interestingly, caspase 3 is able to modulate the function of bcl-2 by cleaving it to a shorter, truncated form which, in contrast to the longer anti-apoptotic form, is pro-apoptotic (Fujita and Tsuruo, 1998). Also, bcl-xL, another anti-apoptotic protein, is cleaved by caspase 3 to a similar truncated pro-apoptotic fragment (Clem et al., 1998).

The immunostaining with all three caspase antibodies was cytoplasmic and mainly diffuse. This is in line with their known cytoplasmic location. Caspases 6 and 8 also showed granular or fragmented cytoplasmic staining. Such staining was only rarely observed with caspase 3. The granular or fragmented cytoplasmic staining many times appeared to result from staining of the fragments suggesting that the concentration of caspases 6 and 8 would be higher in them. The quantity of the granular staining of these two caspases did not, however, associate with the apoptotic index of breast tumours. This might be due to their heterogeneous expression in apoptotic fragments in different tumours, i.e. not all tumours showed staining of apoptotic fragments for caspase 6 to 8 to a similar degree.

In addition to cytoplasmic staining, caspase 8 sometimes also expressed membrane-bound staining. Such staining might be associated with FLICE, the proform of caspase 8, and thus indirectly might reflect a high concentration of the APO-1/FAS receptor complex on the cell membrane and a consequent formation of DISC on tumour cells in some cases. Also nuclear staining was seen, but only with caspase 3. Nuclear caspase 3 staining has previously been described in some non-neoplastic tissues such as type II pneumocytes of the lung, or epithelial cells of the colon or stomach (Krajewska et al., 1997). In non-neoplastic breast epithelial cells, no nuclear staining was, however, observed. We also found a strong association between the expression of all three caspases. This might suggest a mutual regulation of their synthesis in neoplastic breast epithelial cells.

Analysis of the apoptotic index in the breast lesions showed a progressive increase in apoptosis in parallel with the biological aggressiveness of the lesion. The results further show that in situ lesions, there are differences in the apoptotic index depending on the histology or grade of the lesions. Not surprisingly, in situ comedo-type of lesions showed the highest extent of apoptosis followed by solid, cribriform and papillary variants. The lowest apoptotic index was seen in lobular in situ carcinoma. Similarly, high-grade in situ lesions showed a higher extent of apoptosis than intermediate- or low-grade lesions. The results thus suggest that genes that are activated in high-grade lesions may also be responsible for the induction of a higher extent of apoptosis in them. The expression of some cancer genes, such as p53 and c-erbB2, has shown to be different in high-grade in situ lesions compared to low- or intermediate-grade lesions (van de Vijver et al., 1988; O’Malley et al., 1994; Mack et al., 1997). Moreover, comedo-type of in situ carcinomas display less receptor positivity than other in situ carcinomas (Bur et al., 1992; Barnes and Masood, 1990). Oestrogenic stimulation has been shown to inhibit apoptosis through up-regulation of bcl-1 mRNA, and a lack of oestrogen receptors might thus lead to an increased apoptosis in high-grade in situ lesions (Wang and Phung, 1995). In line with this, bcl-2 immunohistochemistry was shown to be reduced in poorly differentiated in situ carcinomas (Maston et al., 1997). Furthermore, there is also an inverse association between the apoptotic index and a positive oestrogen or progesterone receptor status and positive bcl-2 immunohistochemistry in invasive breast carcinomas.
Lipponen et al., 1994; Mustonen et al., 1997). However, we did not observe any association between the expression of caspases and the oestrogen and progesterone receptor status suggesting that their synthesis is not influenced by stimulation of these receptors.

There are several factors which may hamper the use and evaluation of the results obtained by the TUNEL method (reviewed in Soini et al., 1998). Because of this we compared the results with light microscopic morphology and found a statistically significant correlation between both methods. Even though there was a statistically significant correlation, some labelling in the TUNEL method may also be due to DNA fragmentation not necessarily associated with apoptosis.

In conclusion, our results show that the expression of caspases 3, 6 and 8 is increased in parallel with the neoplastic progression of the breast lesions. Their abundant expression in pre-carcinoma-

tous and carcinomatous breast lesions suggests an important role in the execution of apoptosis in malignant breast disease. In accordance with this, their expression was significantly associated with the overall apoptotic index of the lesions.

ACKNOWLEDGEMENTS

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iNOS EXPRESSION, APOPTOSIS AND ANGIOGENESIS IN IN SITU AND INVASIVE BREAST CARCINOMAS

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ABSTRACT

In this investigation we studied the expression of inducible nitric oxide synthase (iNOS) and its association to apoptosis and angiogenesis in 43 in situ and 68 invasive breast carcinomas. Its expression was studied immunohistochemically using a polyclonal iNOS antibody and the staining was evaluated both in tumor and stromal cells. Apoptosis was detected by 3' -end labeling of fragmented DNA (TUNEL method). Vascularisation was detected immunohistochemically using an antibody to FVIII related antigen and calculated microvessel densities (CMVD) were determined. In addition to strong iNOS expression in stromal cells, iNOS positivity was observed in tumor cells in 46.5% of in situ and 58.8% of invasive carcinomas. In invasive carcinomas there were more cases with iNOS positivity both in tumor and stromal cells compared to in situ carcinomas (0.007). The proportion of cases with iNOS positive tumor cells increased in in situ carcinomas from grade 1 to III (20.0%, 46.2% and 75.3%) In invasive ductal carcinomas there were more cases with iNOS positive tumor cells than in in situ carcinomas (P = 0.04). Carcinomas with both iNOS positive tumor and stromal cells had a higher apoptotic index (P = 0.02) and a higher CMVD index (P = 0.02). A high number of iNOS positive stromal cells associated with metastatic disease (P = 0.05). The result show that breast carcinoma cells, in addition to stromal cells, express iNOS and are capable of producing NO. Carcinomas with iNOS positive tumor and stromal cells have a higher apoptotic indices and increased vascularisation suggesting that iNOS contributes to promotion of apoptosis and angiogenesis in breast carcinoma. The association of the number of iNOS positive stromal cells with metastatic disease might be due to stimulation of angiogenesis, resulting in a higher vascular density and consequently a higher probability for tumor cells to invade.

INTRODUCTION

Nitric oxide (NO), a diatomic radical, plays a variety of regulatory functions in vivo. It has diverse physiologic and pathophysiologic roles as a vasodilator, neurotransmitter, antimicrobial effector molecule, and immunomodulator (1). NO is synthesized from the amino acid L-arginine by the NO synthase (NOS) (2). As a free radical, NO is highly reactive and acts in biological systems with other free radicals, molecular oxygen and heavy metals (3). The biological effects of NO are mainly mediated by the products of different NO metabolites (3).

There are three isoforms of NOS: inducible (iNOS, NOS2), endothelial (eNOS, NOS3) and neuronal (nNOS, NOS1) (1). Each isoform is the product of a distinct gene (4). eNOS and nNOS are constitutive, calmodulin-dependent enzymes (eNOS) (4). iNOS is expressed in macrophages, neutrophils, endothelial cells, hepatocytes, cardiac myocytes, chondrocytes and many other cell types (5). It is induced most importantly by cytokines and can generate locally high concentrations of NO for prolonged periods of time (4,6). Ca-independence of iNOS has been questioned with the report of iNOS enzymatic activity dependent on intracellular fluxes of calcium and binding of calmodulin, but in general iNOS is calcium independent (6).

The genotoxicity of NO is due to its reaction with either oxygen or superoxide (7). The intracellular NO quickly forms nitrite and nitrate, ·nitroso-thiols or peroxynitrite (3). NO metabolites can mediate genotoxicity and influence the initiation of cancer by a variety of mechanisms. For instance NO causes DNA damage by nitrosative deamination, DNA strand breakage or DNA modification (e.g. nitration) by peroxynitrite (3,7). These reactions may also be associated with the activation of carcinogenic nitrosamines, initiation of apoptosis and inhibition of DNA repair enzymes or lipid peroxidation-induced DNA damage (6,7).

The effects of NO can be tumor promoting or tumor suppressing. High concentrations of NO can be cytotoxic whereas low concentration may even protect some cell types from damage and apoptosis (3). During the initiation of tumor growth, natural killer cells and macrophages kill tumor cells by NO-mediated mechanism (5). However, NO may also suppress the anti-tumor defense, promote tumor angiogenesis and blood flow in the tumor neoangiogenesis and enhance tumor growth, invasion and metastasis (5).
Table 1. The apoptotic index, vascular density and iNOS immunohistochemistry in different breast lesions

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Apoptosis %</th>
<th>CMVD</th>
<th>iNOS in tumor cells</th>
<th>iNOS in stromal cells</th>
<th>Sum scores for iNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 1 2 3 0 2 3 1</td>
<td>0 1 2 3 0 2 3 1</td>
<td>0 1 2 3 0 2 3 1</td>
</tr>
<tr>
<td><strong>In situ carcinomas</strong></td>
<td>0.70 ± 0.79</td>
<td>13.1 ± 6.3</td>
<td>23</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>Grade I</td>
<td>0.29 ± 0.23</td>
<td>13.0 ± 6.6</td>
<td>12</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Grade II</td>
<td>0.85 ± 0.87</td>
<td>10.4 ± 6.1</td>
<td>7</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Grade III</td>
<td>1.00 ± 0.92</td>
<td>15.6 ± 5.3</td>
<td>4</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td><strong>Invasive carcinomas</strong></td>
<td>1.45 ± 1.24</td>
<td>14.9 ± 7.6</td>
<td>28</td>
<td>32</td>
<td>7</td>
</tr>
<tr>
<td>Ductal grade I</td>
<td>0.71 ± 0.76</td>
<td>14.7 ± 4.6</td>
<td>3</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Ductal grade II</td>
<td>1.47 ± 1.40</td>
<td>15.2 ± 10.4</td>
<td>9</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Ductal grade III</td>
<td>2.14 ± 1.07</td>
<td>13.1 ± 4.9</td>
<td>8</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Lobular</td>
<td>0.58 ± 0.48</td>
<td>19.4 ± 8.4</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Others</td>
<td>0.54</td>
<td>9.7</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

NO exhibits contradictory effects on the regulation of apoptosis. It has been demonstrated to be both pro- and antiapoptotic. The proapoptotic effects appear to be linked to the pathophysiological condition where the induction of iNOS is associated with high concentrations of reactive nitrogen metabolites (8). Cell protection is associated with the upregulation of several protective proteins such as cyclo-oxygenase-2 or heme-oxygenase-1 (9). Typical findings in NO mediated apoptosis include accumulation of tumor suppressor protein p53, caspase activation, chromatin condensation and DNA fragmentation (3,8,9).

In human primary breast cancers relatively high iNOS immunoreactivity have been noted in stromal cells and the presence of stromal reactivity appears to correlate with tumor grade (10). However, in another study iNOS positivity was predominantly found in the tumor cells associated positively with the presence of axillary lymph node metastasis (11). In breast cancer high extent of apoptosis is usually associated with poor prognosis and more apoptosis is seen in tumors of high grade (12,13,14). The expression of iNOS in relation of apoptosis has not been previously studied in different types of breast cancers. In this study we evaluated the immunohistochemical distribution of iNOS in *in situ* and invasive breast cancers and its relation to the apoptotic index, as determined by the TUNEL method. The sections were also studied immunohistochemically for vascular density using FVIII antibody to see whether changes in the expression of iNOS could influence tumor angiogenesis.

**MATERIALS AND METHODS**

A total of 111 breast lesions consisting of 43 *in situ* ductal carcinomas, 56 ductal invasive carcinomas, 10 lobular invasive carcinomas, one mucinous and one medullary carcinoma were collected from the files of the Department of Pathology, University of Oulu. *In situ* carcinomas consisted of 15 low-grade (5 papillary, 3 solid and 7 cribriform), 13 intermediate (1 papillary, 8 solid and 4 cribriform) and 15 high-grade (1 cribriform and 14 comedo-type) lesions. In invasive ductal carcinomas there were 9 well (grade I), 24 moderately (grade II) and 23 poorly differentiated (grade III) tumors. The material had been fixed in neutral formalin and embedded in paraffin. The diagnosis of all the cases were based on light microscopic examination by the conventional hematoxylin and eosin stain (15,16). The grading of the ductal invasive carcinomas were made according to Elston and Ellis (16) and the grades of the *in situ* lesions by Holland et al (17).

The TNM-classification was available in 104 cases. There were 43 T1S, 45 T1, and 16 T2, tumors. 34 cases contained nodal metastases (N1,3) and distant metastases (M1) were present in four cases. Mean follow-up time was 5.8 years (range 0 – 20 years). In 32 cases the cancer relapsed. Information about estrogen and progesterone receptors were available for 63 cases. Values > 10 fmol were considered positive.

**IMMUNOHISTOCHEMICAL STAININGS**

Five-µm paraffin sections were cut from the specimens and placed on SuperFrost®Plus glass slides (Menzel-Gläser, Germany). Immunostainings with iNOS antibodies were performed as follows. Paraffin sections were soaked in xylene to remove paraffin and rehydrated in graded alcohol series. The sections were heated in a microwave oven in 10 mM citric acid monohydrate, pH 6.0 for 10 minutes and then cooled properly at room temperature. The endogenous peroxidase was consumed by immersing the sections in 3% hydrogen peroxide in absolute methanol for 15 minutes. Two different primary antibodies were used: a rabbit polyclonal (dilution 1:200, Santa Cruz
Table 2. iNOS expression in tumor cells of in situ and invasive ductal carcinomas. Invasive carcinomas express significantly more cytoplasmic iNOS positivity than in situ carcinomas (P = 0.05).

<table>
<thead>
<tr>
<th></th>
<th>iNOS negative cases</th>
<th>iNOS positive cases</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>In situ carcinomas</td>
<td>23</td>
<td>20</td>
<td>43</td>
</tr>
<tr>
<td>Invasive ductal carcinomas</td>
<td>20</td>
<td>36</td>
<td>56</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>56</td>
<td>99</td>
</tr>
</tbody>
</table>

Biotechnology) and a mouse monoclonal dilution 1:60, Transduction Laboratories, Lexington) iNOS antibody which both were incubated 60 minutes at room temperature. With these two antibodies Histostain™-PLUS BULK KIT (Zymed Laboratories Inc., South San Francisco, CA) was used. The color was developed by aminoethyl carbazole (AEC) substrate solution (Zymed Laboratories Inc.). The sections were counterstained in Meyer’s hematoxylin followed by 2% ammonia water washing, whereafter the slides were mounted with Immu-Mount (Shandon, Pittsburgh).

Negative control slides were prepared from the same tissue blocks. Instead of using the primary antibody we used PBS. In addition, in seven cases with clear polyclonal iNOS positivity, an absorption test was conducted. Before application to the slides, antibody binding to antigen was neutralized by 2 hours’ pre-absorption at room temperature with a five-fold excess of blocking peptide (Santa Cruz Biotechnology, Inc.) to polyclonal iNOS antibody. Macrophages and neutrophils labeled very strongly (++++) in every slide and they served as an internal positive control for the immunostaining.

The intensity of iNOS immunostainings was evaluated by dividing the cytoplasmic staining reaction in four groups: 1 = weak, 2 = moderate, 3 = strong and 4 = very strong cytoplasmic staining intensity. The quantity of immunostainings were evaluated as follows: 0 = no positive immunostaining, 1 = < 25 %, 2 = 25 - 50 %, 3 = 50 - 75 %, 4 = > 75 % of tumor cells showing cytoplasmic positivity. A combined score for iNOS immunostainings, based on both qualitative and quantitative immunostaining, was composed by adding the qualitative to the quantitative score. This sum score was then divided in five groups as follows: = 0, + = 1 - 2, ++ = 3 - 4, +++ = 5 - 6, ++++ = 7 - 8.

iNOS staining in stromal cells was evaluated with ×40 objective semiquantiatively and divided in three groups as follows: weak 1 = 0 - 2 positive stromal cells / high power field (HPF); moderate 2 = < 10 positive stromal cells / HPF; strong 3 = > 10 positive stromal cells / HPF.

A combined score for iNOS staining in tumor and stromal cells was also calculated. This sum score was divided in four groups as follows: 1 = 1, 2 = 2 - 3, 3 = 4 - 5, 4 = 6 - 7.

For FVIII related antigen, the immunostaining was performed as follows. The sections were dewaxed in xylene and rehydrated in graded alcohol series. For enzyme predigestion of formalin-fixed tissue, the sections were incubated for 30 minutes at 37 °C in 0.04% pepsin (Sigma Chemical Co, St. Louis) in 0.01 M HCl. The endogenous peroxidase activity was consumed by immersing the sections with 3% hydrogen peroxide in absolute methanol. Non-specific binding was blocked by incubating the slides in 20% fetal calf serum in PBS for 20 minutes. The primary polyclonal antibody for factor VIII (DAKO A/S, Glostrup, Denmark) was diluted 1:250 in PBS and incubated 30 minutes at room temperature. Then a biotinylated secondary anti-rabbit antibody (DAKO A/S) diluted 1:300 in PBS was applied on the sections for 30 minutes, followed by the avidin-biotin-peroxidase complex (DAKO A/S). The color was developed by diaminobenzidine, after which the sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany).

As a positive control we used slides from a highly vascularized tumor. Negative controls consisted of PBS instead of the primary antibody.

Calculated microvessel density (CMVD) were counted from an average of six HPFs with ×40 objective. Any endothelial-cell cluster consisting of two or more cells was considered a single, countable microvessel. In in situ carcinomas two distinct vascular patterns could be seen: a diffuse increase of stromal vascularity between ducts and a dense rim of microvessels adjacent to ducts. At first both of them were counted together. The mean of six counts was calculated and used in statistical analysis. Also the periductal vessel density [1/mm] was evaluated separately. The periductal vessels from five round neoplastic ducts were calculated. This sum was then divided by the sum of the measures around these ducts evaluated using the radius of the ducts (2πr) which was measured by an ocular micrometer.

3’-END LABELING OF DNA IN APOPTOTIC CELLS

In order to detect apoptotic cells, in situ labeling of the 3’-ends of the DNA fragments generated by apoptosis associated endonucleases was used. The 3’-end labeling of DNA was performed using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) with a few modification as previously described (18,19). A positive control consisted of a lymph node with follicular hyperplasia. The sections, after been dewaxed in xylene and rehydrated in ethanol, were incubated in 20 μg/ml Proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) at room
Table 3. Apoptotic index in relation to the iNOS sum score in in situ and invasive carcinoma of the breast. The number of cases with a high apoptosis is significantly higher among cases with a high iNOS sum score (P = 0.02).

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>iNOS Sum score =</th>
<th>Sum score &gt; Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apind &lt; 0.63 %</td>
<td>17</td>
<td>39</td>
</tr>
<tr>
<td>Apind ≥ 0.63 %</td>
<td>7</td>
<td>47</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>86</td>
</tr>
</tbody>
</table>

temperature for 15 minutes. The endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in PBS, pH 7.2. The slides were then treated with terminal transferase enzyme and digoxigenin-labeled nucleotides after which anti-digoxigenin-peroxidase solution was applied on the slides. The color was developed with diaminobenzidine after which the slides were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler).

Cells were defined as apoptotic if the whole nuclear area of the cell labeled positively. Apoptotic bodies were defined as small positively-labeled globular bodies in the cytoplasm of the tumor cells, which could be found either singly or in groups. To estimate the apoptotic index (the percentage of apoptotic events in a given area), apoptotic cells and bodies were counted from 10 high power fields (HPFs) with x40 objective and this figure was divided by the number of tumor cells in the same HPFs.

IMMUNOBLOT ANALYSIS

In order to test the specificity of the two iNOS antibodies, immunoblotting analysis using mouse macrophage lysate (Transduction Laboratories) was performed. According to the manufacture the lysate was prepared from the RAW 264.7 (ATCC# TIB71) cell line. These cells were established from an ascites tumor derived from a male mouse which was injected with the Abelson leukemia virus (A-MuLV). Mouse macrophage cells were stimulated with IFNγ and LPS for 12 hours. The control macrophages were mixed with the electrophoresis sample buffer and boiled for 5 minutes at 95°C. 75 µg of cell protein was applied to a 12% sodium dodecyl sulfate-polyacrylamide gel (20). The gel was electrophoresed for 2.0 h (80 V) at room temperature and the protein was transferred onto Hybond ECL nitrocellulose membranes (Amersham, Arlington Hights, IL) in a Mini-PROTEAN II Cell (Bio-Rad, Hercules, CA). The blotted membrane was incubated with the poly- and monoclonal antibodies to iNOS (dilutions 1:2000 for both antibodies) followed by treatment with secondary anti-mouse and anti-rabbit antibodies (dilutions 1:2000 for both secondary antibodies, Jackson Immunoresearch Laboratories) conjugated to horseradish peroxidase. The proteins were detected by enhanced chemiluminescence system (ECL, Amersham). Cell protein was measured using the Bio-Rad protein assay (Bio-Rad) (21).

STATISTICAL ANALYSIS

SPSS for Windows (Chicago, IL) was used for statistical analysis. The significance of associations were determined using Fisher’s exact probability test, correlation analysis and two tailed t-test. Survival was analyzed by applying the Kaplan-Meyer method with log rank analysis. Probability values ≤ 0.05 were considered significant.

RESULTS

iNOS IMMUNOREACTIVITY

The results of the study are compiled in Table 1. Strong iNOS expression could be seen in stromal macrophages and neutrophils. Also stromal fibroblasts and endothelial cells often expressed cytoplasmic positivity for iNOS. In benign non-neoplastic breast epithelial cells inconsistent expression of iNOS could be seen both in the ductal and acinar structures (Figure 1).

In the whole material 51 (45.9%) cases showed no staining for iNOS in the tumor cells, 49 (44.1%) stained weakly (+), 10 (9.0%) moderately (+++) and one (0.9%) strongly (+++). None of the breast tumors labeled very strongly (++++) with the antibody. The number of positive stromal cells was small in 41 (36.9%), moderate in 46 (41.4%) and high in 24 (21.6%) cases. There was a clear association between tumor cell positivity and high number of positive stromal cells (P = 0.017). The sum score for iNOS immunoreactivity were 1 in 24 (21.6%), 2 in 62 (55.9%) and 3 in 25 (22.5%) cases. None of the cases reached sum score 4.

The distribution of iNOS expression in tumor and stromal cells of in situ carcinomas can be seen in Table 1 (Figure 2A). The number of iNOS positive cases increased from grade I to III (20.0%, 46.2% and 73.3%). There were significantly less iNOS positive cases in grade I than in grades II-III in situ lesions (P = 0.01) and in grade I-II than in grade III in situ lesions (P = 0.01). No significant differences were found when comparing iNOS positivity in stromal cells or the sum scores in different grades of in situ carcinomas (data not shown).

iNOS expression in tumor and stromal cells of invasive carcinomas are compiled in Table 1 (Figures 3A and 4A). No significant difference was found in iNOS positivity in tumor and/or stromal cells between different grades in invasive ductal carcinomas (data not shown). Invasive ductal carcinomas contained more cases with iNOS positive tumor cells than invasive lobular carcinomas (64.3% and 30.0% respectively, P = 0.04).
Table 4. Vascularisation in relation to the iNOS sum score in in situ and invasive carcinoma of the breast. The number of cases with a high vascular density is significantly higher among iNOS positive cases (P = 0.02).

<table>
<thead>
<tr>
<th>Vascular density</th>
<th>iNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sum score = 1</td>
</tr>
<tr>
<td>CMVD &lt; 12.8</td>
<td>16</td>
</tr>
<tr>
<td>CMVD ≥ 12.8</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
</tr>
</tbody>
</table>

iNOS positivity was more often found in tumor cells of invasive ductal carcinomas than in in situ carcinomas (P = 0.05, Table 2). Also the number of positive stromal cells and sum scores were higher in invasive ductal carcinomas (P = 0.03 and P = 0.004 respectively). All different invasive carcinomas had more cases with a very high number of iNOS positive stromal cells than in situ carcinomas (P = 0.03) and they had also higher sum scores (P = 0.006).

All PBS control slides were negative. iNOS immunoreactivity was completely abolished in all slides where the antibody was pre-treated with the blocking peptide in the absorption experiment (Figures 3C and 4C).

APOPTOTIC INDEX

The apoptotic indices are shown in Table 1. The mean apoptotic index was 1.16 ± 1.14 % and median 0.63%. Low grade in situ lesions showed a significantly lower extent of apoptosis (0.29 ± 0.23 %) than intermediate and high grade lesions (0.93 ± 0.88 %) (P = 0.009).

Similarly, low and intermediate grade lesions showed a lower extent of apoptosis (0.55 ± 0.67 %) than high grade in situ lesions (1.00 ± 0.92 %) (P = 0.07). In different grades of ductal invasive carcinomas the apoptotic indices increased with the tumor grade: grade I (0.71 ± 0.76 %) and grade II-III (1.81 ± 1.28 %) (P = 0.02) or grade I-II (1.28 ± 1.30 %) and grade III (2.14 ± 1.07 %) (P = 0.01). There were also significant differences in apoptotic indices between invasive ductal (1.64 ± 1.27 %) and in situ carcinomas (0.70 ± 0.79 %) (P < 0.001) and in situ lesions and all invasive carcinomas (1.45 ± 1.24 %) (P = 0.001).

VASCULAR DENSITY

The results of calculated microvessel densities (VMVD) detected by FVIII-related antigen are shown in Table 1. The mean CMVD was 14.2 ± 7.1 / HPF and median was 12.8 / HPF. There were no significant differences in vascular density between in situ or invasive carcinomas or between different grades of the tumors (data not shown).

In in situ carcinomas the average periductal microvessel density was 10.8 ± 5.8 mm⁻¹ (range 0.8 – 31.2 mm⁻¹, median 10.15 mm⁻¹). The densities in different grades of in situ carcinomas were: low-grade 9.0 ± 4.9 mm⁻¹, intermediate 12.0 ± 7.5 mm⁻¹ and high-grade 10.9 ± 4.8 mm⁻¹. There were no significant differences comparing periductal microvessel densities between grade I and II-III or grades I-II and III (P = 0.22 and P = 0.79 respectively).

ASSOCIATIONS OF iNOS WITH APOPTOSIS, VASCULAR DENSITY, TNM-CLASS, SURVIVAL AND ESTROGEN AND PROGESTERONE RECEPTOR STATUS

In the whole material tumors with a high sum scores (>1) for iNOS had a higher apoptotic index (≥ 0.63 %) (Fisher’s exact test P = 0.02, Table 3). Moderate or high number of iNOS positive stromal cells associated also with higher apoptotic index (P = 0.03) but iNOS positivity in tumor cells alone did not (P = 0.12).

There was a significant association between iNOS positivity in tumor cells and a high (≥12.8) CMVD value (P = 0.04). Also a moderate or high number of positive stromal cells associated with high CMVD index alone (P = 0.05) and as the sum with iNOS positivity in tumor cells (P = 0.02) (Table 4). The periductal microvessel density tended to be higher in iNOS positive (12.3 ± 6.3 mm⁻¹) than in negative (9.1 ± 5.0 mm⁻¹) cases (P = 0.08).

There was no difference in iNOS positivity in tumor cells between invasive (T1,2) and in situ (T0) carcinomas (P = 0.14) but in invasive carcinomas there were more cases with a high number of positive stromal cells (P = 0.05). Also the sum of iNOS positivity in tumor and stromal cells associated clearly with invasive tumors (P = 0.004). A high number of iNOS positive stromal cells associated with nodal and/or distant metastasis (P = 0.05). The iNOS positivity in tumor cells or the sum of iNOS positivity in tumor and stromal cells did not correlate with nodal and/or distant metastases (data not shown).

iNOS positivity in tumor or stromal cells did not associate with survival, estrogen or progesterone receptor positivity (data not shown). However, the sum of iNOS positivity in tumor and stromal cells correlated with the progesterone receptor positivity (P = 0.02).

COMPARISON OF THE POLYCLONAL WITH THE MONOCLONAL iNOS ANTIBODY

To study the reliability of polyclonal iNOS antibody, we evaluated inducible NOS activation with a monoclonal iNOS antibody from 53 samples. According to the results, there was a strong positive correlation between iNOS positivities with the polyclonal and monoclonal iNOS antibodies (P = 0.002) (Figures 3B and 4B).
IMMUNOBLOT ANALYSIS

To further test the reliability of the polyclonal and monoclonal iNOS antibodies an immunoblot analysis with control macrophages was performed with both of them. A positive bands corresponding to the 130 kDa molecular weight of iNOS protein could be detected with both antibodies (Figure 5).

DISCUSSION

This study was undertaken to investigate the expression of iNOS in in situ and invasive carcinomas of the breast. Since NO is known to influence apoptosis and angiogenesis we also studied the extent of apoptosis and vascular density in the breast lesions.

A previous study has demonstrated that in human breast tumors iNOS is mainly expressed in stromal cells and not in tumor cells and that its stromal presence correlates with tumor grade (10). However, a study with ZR-75-1 human breast cancer cells revealed that these cells contain iNOS and produce spontaneously NO (22). While we also observed strong expression of iNOS in stromal macrophages and neutrophils in our study, also a proportion of tumor cells displayed clear positivity. In fact 58.8 % of all invasive and 46.5 % of in situ lesions displayed some iNOS positivity in a proportion of the tumor cells. The results thus suggest that, in addition to stromal macrophages and neutrophils, breast carcinoma cells also contain detectable levels of iNOS and are thus capable of producing NO. In fact, the results of a recent study by Dueñas-Gonzales et al. are in keeping with our results (11). To substantiate the findings, we immunostained a part of the lesions with a monoclonal iNOS antibody. Immunostaining with this antibody also showed the presence of iNOS in breast carcinoma tumor cells and the results were thus consistent with the results obtained by the polyclonal iNOS antibody.

In other types of epithelial tumors iNOS positivity has been reported in tumor cells of prostate carcinoma (23), gynecological carcinoma (24), colon carcinoma (25,26) and transitional cell carcinoma of the bladder (27). Recently, strong iNOS synthesis was also discovered in malignant mesothelioma (28). These results are consistent with our findings and suggest that in addition to other neoplasms, also breast carcinoma cells are able to modulate NO synthesis.

In in situ lesions of the breast there were significantly more cases with iNOS positive tumor cells in high grade than in low grade tumors. iNOS positivity in tumor cells increased in ductal lesions from in situ
carcinomas to invasive. Invasive carcinomas had also more cases with a very high number of iNOS positive stromal cells than in situ carcinomas. These results suggest that there is an up-regulation of iNOS positivity along with the biological aggressiveness of the breast lesions. Increased iNOS activity has been positively correlated with the degree of malignancy also in gynecological tumors (24). In colon carcinomas the expression of iNOS remains controversial while both decreased (25,29) and increased (26) expression of iNOS has been reported with increasing tumor stage.

NO has been reported to be both pro- and anti-apoptotic. It has been shown to inhibit apoptosis in several cell types, including endothelial cells (30), hepatocytes (31), lymphocytes (32), leukocytes (33) and eosinophils (34). NO induces apoptosis in various cells, including macrophages (35), pancreatic β-cells (36) and thymocytes (37). There is also a study suggesting that low concentration of NO inhibits apoptosis, but high concentrations of NO induces apoptosis in human venous endothelial cells (38). The role of NO in apoptosis appears to be cell type specific and depends on the NO concentration being produced.

In our material there was a gradual increase in apoptotic index from low grade in situ carcinomas to high grade invasive ductal carcinomas with a coexistent up-regulation of total iNOS. A consequent increased production of NO might thus be one reason for the accelerated apoptosis. There were significantly more cases with a high apoptotic index showing iNOS positivity in tumor and stromal cells than in cases with a low apoptotic index. These results suggest that NO, produced by iNOS in breast tumor and in stromal cells, could be an additional factor participating in the enhancement of apoptosis in them.

There are several reports concerning the role of NO in angiogenesis. In vitro studies have demonstrated that NO donors increase and iNOS inhibitors attenuate DNA synthesis, proliferation and migration of coronary venular endothelial cells (39). On the other hand, both NO donors and iNOS inhibitors have no effect on the fibroblast growth factor induced proliferation and migration of endothelial cells (39). In vivo studies have shown that NO donors potentiate and iNOS inhibitors attenuate angiogenesis in rabbit (39) and rat cornea (40). Also iNOS-transfected human colon adenocarcinoma DLD-1 cells had higher vessel density and growth rate in vivo than parental cells (41). In murine mammary tumor model the data suggest that NO is a key mediator of C3L5 tumor-induced angiogenesis being reduced in NOS inhibitor treated mice (42). However, also controversial results are obtained (43,44,45). These findings suggest that NO partially mediates angiogenesis and that the
involvement of NO is both tissue and/or growth stimuli dependent.

We studied tumor angiogenesis with an antibody to FVIII related antigen and compared vascular density with iNOS expression. Tumors with iNOS positivity in tumor and/or stromal cells had increased vascular densities in the whole material. Also in \textit{in situ} carcinomas where vascular densities could also be determined in the vicinity of neoplastic ducts, the vascular densities tended to be higher in iNOS positive cases. These results suggests that local NO production by iNOS in breast carcinoma cells are able to modulate angiogenesis. NO production by stromal cells enhances this effect even more.

iNOS expression may play a role in human cancer progression. While a few reports indicate that the presence of NO in tumor cells or their microenvironment is detrimental for tumor-cell survival, a large body of evidence suggests that NO promotes tumor progression (6,46,47). In murine mammary adenocarcinoma increased NO production has been shown to promote tumor-cell invasiveness (48). We tested whether the iNOS positivity in breast cancer influences tumor growth measured by TNM-status or patient survival. There were no association between iNOS positivity in breast tumor cells alone and TNM-class. However, there were more cases with iNOS positive tumor and stromal cells within invasive tumors (T1+4) compared to \textit{in situ} tumors (T1+). Also nodal and distant metastases increased in cases with iNOS positive stromal cells. These results indicate that in breast tumors NO produced by stromal cells enhance tumor growth, invasiveness and metastatic ability. A part of this effect might be mediated through an increased angiogenesis caused by iNOS, which, on one hand, would enhance the nutrition of the tumor cells, on the other hand, through increased vessel density, would make more blood vessels available for tumor cells to invade.

There are no previous \textit{in vivo} or \textit{in vitro} studies on iNOS expression and hormone receptors in breast cancer. However, eNOS and nNOS have been shown to be expressed only in estrogen receptor positive breast cancer cell lines (49). Studies with other tissues or cell lines have revealed that high amounts of estradiol induces iNOS production in rats aortas (50) and in human umbilical vein endothelial cells (51). However, physiological concentrations of 17β-estradiol inhibit iNOS production in murine macrophage cell line (52). Also progesterone has been shown to inhibit iNOS production in murine macrophages (53). In our material there were no association between iNOS positivity and estrogen receptor positivity. However, the sum of iNOS positivity in tumor and stromal cells correlated with the progesterone receptor positivity. The results indirectly suggest that hormone receptor status and hormonal stimulus may influence iNOS expression in breast carcinoma cells. What effect certain hormone stimulus eventually has seems to be dependent on cell type and hormone concentration.

At the present time the role of NO in tumor biology is still poorly understood. According to previous reports NO seems to have a double-edged role in tumor progression, apoptosis and angiogenesis. iNOS response to hormone stimulus varies also depending on cell type and activity. The effect of iNOS depends on the concentrations of NO being produced and the local environment in different tumor and cell types. Our results show that in addition to stromal cells iNOS is expressed in neoplastic cells of breast carcinoma. In ductal lesions the iNOS positivity in tumor cells increased from \textit{in situ} carcinomas to invasive carcinomas. Invasive carcinomas had also more cases with a very high number of iNOS positive stromal cells than \textit{in situ} carcinomas. The results thus show that there is an up-regulation of iNOS positivity along with the biological aggressiveness of the breast lesions. NO produced by iNOS in breast tumor and stromal cells seems to enhance apoptosis. On the other hand, NO production by tumor cells and stromal cells increases tumor vascularisation and possibly through this effect also enhance tumor growth and metastatic ability.

ACKNOWLEDGEMENTS

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MnSOD expression is less frequent in tumour cells of invasive breast carcinomas than in *in situ* carcinomas or non-neoplastic breast epithelial cells.

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**Summary**

Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme capable of neutralizing superoxide anion molecules. In previous studies it has been suggested to suppress both tumour proliferation and apoptosis. In this study we investigated 46 invasive, 50 *in situ* and 19 benign hyperplastic breast lesions for its immunohistochemical expression. To study cell proliferation, apoptosis and their association with MnSOD expression we immunostained the neoplastic breast lesions with a monoclonal antibody to Ki-67 and determined the extent of apoptosis by the TUNEL method in them. 24/46 (52%) of the invasive ductal carcinomas, 41/50 (82%) of the *in situ* and 15/19 (79%) of the benign hyperplasias expressed positivity for the MnSOD protein. There were significantly more MnSOD positive cases in *in situ* carcinomas and in benign hyperplasias than in invasive carcinomas (p=0.0099 and p=0.039, respectively). Positivity was also more frequently found in non-neoplastic ductal and acinar epithelial cells than in invasive carcinomas. On the other hand, neoplastic epithelial cells of invasive and *in situ* carcinomas showed more often strong positivity than the epithelial cells of benign hyperplasias or non-neoplastic epithelium. In breast carcinomas MnSOD positive cases tended to show a lower proliferation index, but the association did not reach statistical significance (p=0.066). Apoptosis did not associate with MnSOD expression. The lower frequency of MnSOD positive cases in invasive breast carcinomas suggests that the lack of its expression might contribute to the development of an invasive breast carcinoma phenotype, and that it could in this way operate as a tumour suppressor gene as previously suggested. The lack of its expression might also contribute to an increased tumour cell proliferation as suggested by the tendency for an inverse association between the Ki-67 and MnSOD expression.

**KEY WORDS—**MnSOD; apoptosis; Ki-67; breast cancer

**Introduction**

Reactive oxygen species (ROS) play a pivotal role in many physiologic reactions, such as signal transduction and apoptosis, and also participate in carcinogenesis (1,2). Cells have efficient antioxidant capacity against ROS; most important of these mechanisms being intracellular antioxidant enzymes (AOEs). The enzymes may have an important role also in cell proliferation (3,4), tumor invasion (5) and drug resistance of malignant cells (6). With the exception of glutathione-S-transferase, an enzyme closely associated with cell defence and detoxification (6), AOE s in human malignant tumors are poorly characterized. Superoxide dismutases decompose superoxide radicals to hydrogen peroxide; hydrogen peroxide and other peroxides of the cell are then consumed by multiple enzymes such as catalase and glutathione peroxidase (7).

There are three superoxide dismutases (SODs) in human tissues, MnSOD in the mitochondria, CuZnSOD in the cytosol and extracellular SOD in the extracellular matrix (8,9). MnSOD is considered to be one of the most important antioxidant enzymes both against endogenous and exogenous superoxide (10,11). The basal expression of MnSOD is usually low, often hardly detectable, but the enzyme is induced by hyperoxia (10), irradiation (12), cytokines, such as tumor necrosis factor (13), interleukins (14) and interferon γ (15), and changes in the cellular redox state (16). Recently upregulation of MnSOD has also been shown to occur in response to the exposure to various cytotoxic drugs at least in human lung adenocarcinoma cells and human granulocytes (17,18). Several but not all studies have also emphasized the importance of superoxide dismutase in protection of cells and tissues against hyperoxia, cytokines and possibly cytotoxic drugs both *in vitro* and *in vivo* (13, 19-23). For instance, tracheal insufflation of tumor necrosis factor alpha (TNFα) and subsequent induction of MnSOD has rendered adult rats more resistant to oxygen toxicity (24) and induction or transfection of MnSOD gene appears to block TNFα- and/or oxidant- mediated cytotoxicity at least in fibroblasts, breast cancer cells, hematopoietic cells and lung adenocarcinoma cells (20, 25,26).

Several studies have suggested that the level of MnSOD is low in tumor cells (4). Furthermore MnSOD activity has been shown to correlate with the degree of differentiation of nonmalignant and malignant cells so.
that in general more differentiated cells have higher MnSOD activity (4,5). Tumor cells overexpressing MnSOD after transfection of the MnSOD gene have been shown to have lower mitotic rate as demonstrated by proliferating cell nuclear antigen staining. Therefore MnSOD gene has also been suggested to be tumor suppressor gene (5). On the other hand recent studies have also documented high levels of MnSOD in many malignant tumors such as mesothelioma, colon carcinoma, thyroid carcinoma and tumors of neural origin (27-31). The role of MnSOD in tumor cells and in their resistance in vivo is still unclear since most conclusions have been obtained with tumor cells with transfected MnSOD. To further clarify the role of MnSOD in malignant tumors, we investigated the immunohistochemical distribution of MnSOD in a set of breast invasive and in situ carcinomas and compared the reactivity with that found in benign breast hyperplasias and non-neoplastic breast epithelial cells. Given that the level of MnSOD has been shown to inversely correlate with the mitotic rate of malignant cells in vitro, additional experiments were conducted to assess the correlation of MnSOD with the proliferation of the tumor in vivo. Since MnSOD has been suggested to abrogate apoptosis (32) we also determined the extent of apoptosis in the cases by using the TUNEL method.

Materials and methods

Study material
The study set consisted of 46 invasive and 50 in situ lesions and 19 benign hyperplasias which were obtained from the files of the Department of Pathology, Oulu University Central Hospital between 1995 and 1998. All the material had been fixed in 10 % buffered formalin and embedded in paraffin. The histological diagnosis and the grades of the invasive carcinomas were according to the criteria of Elston and Ellis (33), and the grades of the in situ lesions by Holland et al. (34).

Immunohistochemical stainings
A polyclonal rabbit antibody for human MnSOD was a gift from Professor J.D. Crapo (National Jewish Medical Center, Denver, Colorado) (27). Tumour cell proliferation was studied with a monoclonal mouse anti-human Ki-67 antibody (Zymed, San Francisco, CA, USA). Five μm thick sections were cut from representative tumour blocks. The sections were first deparaffinized in xylene and rehydrated in descending ethanol series. In order to enhance the Ki-67 staining reaction, the sections were incubated in 10 mM citrate buffer (pH 6.0) and boiled for 2 min at 850 W and after that 8 min in 350 W. Endogenous peroxidase activity was eliminated by incubation in 0.1 % hydrogen peroxide in absolute methanol for 10 minutes. The concentration of the primary anti-MnSOD antibody was 1:1000 and for anti-Ki-67 1:50. For anti-MnSOD the immunostaining was performed using the Histostain-Plus Bulk Kit (Zymed Laboratories Inc, South San Francisco) and the chromogen used was AEC (Zymed Laboratories Inc). For anti-Ki-67 the avidin-biotin-peroxidase complex method was used, the color was developed using 3,3'-diaminobenzidine. The sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). Replacement of the primary antibodies with phosphate buffered saline (PBS) at pH 7.2 was used as a negative control.

Figure 1. MnSOD staining in non-neoplastic epithelial cells. The acinar cells display strong cytoplasmic positivity for MnSOD. A heavy inflammatory infiltrate can be seen around the acinar structures. (immunoperoxidase stain)

Figure 2. In a in situ ductal carcinoma strong cytoplasmic MnSOD immunostaining can be seen in myoepithelial cells surrounding the neoplastic ductal structures. Occasional positivity can be seen in some carcinoma cells. (immunoperoxidase stain)
The immunostaining results for MnSOD were evaluated as follows. The intensity of the immunostainings with all the antibodies was evaluated by dividing the staining reaction in four groups:

1 = weak cytoplasmic staining intensity,
2 = moderate cytoplasmic staining intensity,
3 = strong cytoplasmic staining intensity, and
4 = very strong cytoplasmic staining intensity.

The quantity of the immunostaining was evaluated as follows:

0 = No positive immunostaining,
1 = ≤ 25% of tumor cells showing cytoplasmic positivity,
2 = 25-50% of tumor cells showing cytoplasmic positivity,
3 = 50-75% of tumor cells showing cytoplasmic positivity, and
4 = > 75% of tumor cells showing cytoplasmic positivity.

A combined score for the immunostaining, based on both qualitative and quantitative immunostaining was composed by adding both the qualitative and quantitative score which was then divided in four main groups:

- = no immunostaining; score 0,
+ = weak immunostaining; scores 1-2,
++ = moderate immunostaining, scores 3-4,
+++ = strong immunostaining, scores 5-8.

For Ki-67, the staining was evaluated semiquantitatively and divided in four groups:

Weak staining = less than 5% of cell nuclei positive
Moderate staining = 5-10% of cell nuclei positive
Strong staining = 10-50% of cell nuclei positive
Very strong staining = Over 50% of cell nuclei positive

**Determination of apoptosis**

In order to detect apoptotic cells, in situ labeling of the 3'-ends of the DNA fragments generated by apoptosis-associated endonucleases was performed using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD, USA) as previously described (35,36). To estimate the apoptotic index (the percentage of apoptotic events in a given area), apoptotic cells and bodies were counted in 10 high power fields (HPPs) and this figure was divided by the number of tumor cells in the same HPPs.

**Statistical analysis**

SPSS for Windows (Chicago, IL, USA) was used for statistical analysis. The significance of the associations were determined using Fisher's exact probability test, correlation analysis and two-tailed t-test. Probability values p ≤ 0.05 were considered statistically significant.

**Results**

**Non-neoplastic tissue**

Granular MnSOD immunoreactivity could be seen both in ductal and lobular breast epithelial cells. The immunoreactivity was, however, inconsistent and was more strongly present in non-neoplastic glands which had been entrapped in tumour tissues (Figure 1). Also, epithelial cells which were surrounded by inflammation expressed relatively strong immunoreactivity. In addition to the epithelial cells, also myoepithelial cells expressed MnSOD immunopositivity. Positivity could be seen both around non-neoplastic ductal structures as well as around in situ lesions (Figure 2).
MnSOD in breast lesions

Figure 5. A ductal in situ carcinoma of the comedo type showing strong MnSOD positivity in a proportion of the tumour cells. Positivity can especially be seen in the central areas of the neoplastic ducts. (immunoperoxidase stain)

Fibroblasts and granulocytes among the neoplastic stroma frequently expressed strong granular cytoplasmic immunoreactivity for MnSOD (Figure 3). Also tissue histiocytes and endothelial cells expressed MnSOD. In one case, a focal area of fat necrosis was seen showing strong positive staining in macrophages involved in the lesion. Positivity was also seen in smooth muscle cells of small vascular structures. Lymphocytes and lymphatic cells in germinal centers were, however, negative.

Neoplastic tissues and hyperplasias

The results on the staining of breast carcinomas, in situ breast lesions, hyperplasias and normal epithelial lesions are shown in Table 1. 24/46 (52%) of the invasive carcinomas expressed positivity for MnSOD in tumour cells (Figure 4). In many cases the stromal cells expressed variable, but usually strong positivity for MnSOD. Invasive grade II-III lesions tended to have more MnSOD-negative cases than invasive grade I lesions (p=0.07). Invasive carcinomas more often expressed negative staining in tumour cells compared to in situ carcinomas (p=0.0009) or sections with hyperplasias (p=0.039) or normal breast epithelium (p=0.00005).

41/50 (82%) of the in situ carcinomas were positive (Figure 5). There were significantly more cases expressing positivity in in situ carcinomas than in invasive carcinomas (see above) but no clear differences could be seen in the immunostaining between the different grades of in situ lesions. In in situ lesions the immunostaining was many times stronger in the central areas of the neoplastic ducts, and in comedo carcinomas, strong immunoreactivity was many times seen in the vicinity of the central necrotic areas. In situ and invasive carcinomas expressed more often cases with strong MnSOD immunoreactivity than hyperplasias (p=0.034).

MnSOD, proliferation and apoptosis

53% of the invasive and in situ carcinomas showed weak or moderate Ki-67 expression and 47% strong expression. There was a marginal inverse association between MnSOD and Ki-67 expression in the carcinomas but the association did not reach statistical significance (p=0.066). The apoptotic index in in situ carcinomas was 0.69±0.1 % and in invasive carcinomas 0.76±0.21 % (p=0.74). Cases with a moderate or strong M.B-index showed significantly more apoptosis (0.82±0.93 %) than cases with a low index (0.32±0.28 %) (p=0.003). There was no association between MnSOD expression and apoptosis in invasive and in situ carcinomas (p=0.382).

Discussion

In this study we investigated the immunohistochemical expression of MnSOD in a set of invasive and preinvasive breast carcinomas and compared the staining with that found in benign epithelial hyperplasias and in sections from normal breast tissues. According to the results its expression is less frequent in neoplastic epithelial cells of invasive breast carcinomas than in preinvasive or non-neoplastic breast epithelium. There were no quantitative differences in the MnSOD expression between in situ carcinomas and non-neoplastic epithelium. On the other hand, carcinoma cases more often showed stronger staining in tumour cells than benign epithelial lesions.

<table>
<thead>
<tr>
<th>Table 1. MnSOD immunoreactivity in invasive and in situ carcinomas, benign hyperplasias and normal epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive Carcinoma</td>
</tr>
<tr>
<td>grade 1             grade 2            grade 3</td>
</tr>
<tr>
<td>Negative     0               11             11</td>
</tr>
<tr>
<td>Weak         1               4               4</td>
</tr>
<tr>
<td>Moderate     2               4               3</td>
</tr>
<tr>
<td>Strong       1               3               3</td>
</tr>
<tr>
<td>Total        22              29              29</td>
</tr>
<tr>
<td>In situ carcinoma</td>
</tr>
<tr>
<td>grade 1             grade 2            grade 3</td>
</tr>
<tr>
<td>Negative     1               2               1</td>
</tr>
<tr>
<td>Weak         2               8               3</td>
</tr>
<tr>
<td>Moderate     8               18              12</td>
</tr>
<tr>
<td>Strong       1               2               0</td>
</tr>
<tr>
<td>Total        18              20              11</td>
</tr>
<tr>
<td>Normal epithelium</td>
</tr>
<tr>
<td>Hyperplasia</td>
</tr>
<tr>
<td>Weak         3               4               1</td>
</tr>
<tr>
<td>Moderate     1               2               6</td>
</tr>
<tr>
<td>Strong       0               1               0</td>
</tr>
<tr>
<td>Total        4               7               8</td>
</tr>
</tbody>
</table>
The expression of MnSOD in various tumors may be tissue and tumor specific. Although many studies have shown low MnSOD in malignant cells and especially in cells with high proliferation (4), MnSOD is constantly high at least in renal tumors (37), pleural mesothelioma (27), glioblastomas (28) and in colon tumors (29). On the other hand, MnSOD is low in prostate carcinomas (38). Marklund analyzed MnSOD activity in a large number of various tumor cell lines, and in that particular study malignant mesothelioma cells were the only one which showed high MnSOD activity (9).

Surprisingly, no studies have been conducted on the expression of MnSOD in non-cancerous and cancerous human breast tissues. Blanchet et al., however, showed by epinephrine autooxidation assay that breast carcinoma tissues contain a variable but usually a higher activity of superoxide dismutases than normal breast tissue (39). Even though we found a decreased immunohistochemical expression of MnSOD in neoplastic epithelial cells of invasive carcinomas, the results of Blanchet et al. are not contradictory to our findings. Breast tumor tissues many times contain reactive non-neoplastic cells, such as fibroblasts, granulocytes, histiocytes and endothelial cells, which are MnSOD positive, and which may increase the total amount of MnSOD in tumor tissues even though the tumor cells themselves would be negative. In fact, such reactive MnSOD positive cells were frequently found in large numbers in our breast carcinoma samples. Moreover, the level of other superoxide dismutases, such as CuZnSOD and extracellular SOD, may further influence the total SOD activity in breast tissues.

Our results showing a decreased MnSOD expression in invasive carcinoma tumour cells compared to in situ carcinomas or nonmalignant epithelial cells suggests that a decreased MnSOD expression is associated with the development of an invasive phenotype of the neoplastic breast epithelial cells. This is in line with the previous results suggesting that MnSOD might operate as a tumor suppressor gene (3). The result is also in agreement with a finding that MnSOD upregulates the expression of the protease inhibitor maspin which inhibits tumor cell motility and invasion (40). Downregulation of MnSOD might thus lead to increased motility of the cells and to an increased invasive potential in breast carcinoma. In a recent study on breast carcinomas, Ambrosone et al. showed that a polymorphism in the MnSOD gene which changes its mitochondrial targeting sequence increases the risk of breast cancer. This finding indicates that oxidative damage may be an important pathogenetic factor in the evolution of breast carcinoma and also stresses the putative importance of MnSOD's function in the development of breast carcinoma (41).

In cell line studies on human breast cancer cells it has been shown that overexpression of MnSOD results in abrogation of proliferation (42). Previous cell line studies have shown that MnSOD may also suppress apoptosis (25,43). In our recent study on mesotheliomas we found that proliferation was inversely associated with MnSOD expression (44). We also showed that a mesothelioma cell line expressing high levels of MnSOD showed resistance to epirubicin-induced apoptosis compared to another cell line showing a lower MnSOD expression (44). According to the present results MnSOD expression might abrogate proliferation in breast carcinomas in vivo. On the other hand, breast carcinomas did not show any significant association between spontaneous apoptosis and MnSOD expression. These results indicate that in breast carcinomas the influence of MnSOD on proliferation might be more important for tumor evolution than its influence on apoptosis.

Even though neoplastic breast cells, especially invasive tumors, showed a quantitatively lower expression of MnSOD, 52 % of invasive carcinomas and 82 % of in situ lesions were positive for MnSOD. This suggests that a majority of neoplastic breast epithelial cells are still capable of synthesizing MnSOD and have retained their ability to counteract the damage caused by ROS. The fact that breast carcinomas contained cases with strong MnSOD expression is probably an indication of the tumor cells to counteract oxidant damage caused by the hypoxic conditions and ROS production induced by the surrounding inflammation in tumour tissues. In conclusion, the results show that invasive breast carcinomas less frequently express MnSOD than preinvasive in situ breast lesions or benign hyperplasias. Its reduced expression is in line with the suggested tumour suppressor nature of MnSOD. According to the results the lack of MnSOD expression in invasive breast carcinomas might also promote tumour growth through increase of proliferation of the tumour cells. Future studies with larger clinical materials will show whether MnSOD has any prognostic significance in the progression of breast carcinoma and whether it can be used as a prognostic marker.

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References


