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LATE DERMAL EFFECTS OF
BREAST CANCER
RADIOTHERAPY

FACULTY OF MEDICINE,
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UNIVERSITY OF OULU

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RIITTA RIEKKI

**LATE DERMAL EFFECTS OF BREAST
CANCER RADIOTHERAPY**

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Abstract

Radiotherapy is used in the treatment of breast cancer in order to reduce local recurrence rate. However, radiation is known to cause both acute and delayed side-effects on normal tissues. A common late complication of radiotherapy is fibrosis of skin and other organs. Fibrosis has been described as excessive accumulation of extracellular matrix components, especially collagens.

Collagens are a group of extracellular matrix proteins that provide connective tissues with tensile strength. Type I and III collagens are the major structural proteins in skin. Alterations in collagen synthesis occur in various pathological conditions, during ageing and in association with diverse medical therapies. Collagens are degraded by matrix metalloproteinase enzymes (MMPs). The activity of MMPs is restrained by their specific tissue inhibitors (TIMPs).

Elastic fibres constitute about 2–4% of skin dry weight. Despite their low quantity, elastic fibres are responsible for the resilient and elastic properties of skin. Dermal elastic fibres may be affected by intrinsic ageing, by extrinsic reasons such as photodamage and in several connective tissue diseases.

The effect of radiotherapy on human skin type I and III collagen synthesis was investigated in a group of women who had been treated for breast cancer surgically and with radiotherapy. The levels of MMP-9, MMP-2/TIMP-2 complex, TIMP-1 and TIMP-2 in irradiated skin were also analysed. The effect of radiotherapy on elastic fibres was analysed using skin samples. The physio-mechanical properties of radiotherapy-treated skin were studied using ultrasound and elastometer devices, and compared with those of non-treated skin.

In addition, skin samples were stained for haematoxylin-eosin, tenascin and mast cells. Factor VIII immunostaining was performed to visualize dermal blood vessels. Wound regeneration in irradiated skin was also studied using suction blister as a model.

The synthesis of type I and III collagens was markedly increased as a result of radiotherapy. An increased amount of cross-linked type I collagen was detected in irradiated skin, and collagen turnover was also increased in irradiated skin. No difference in the amount or structure of the elastic fibres could be found between radiotherapy-treated and non-treated skin. A slight increase of skin thickness and stiffness was found in irradiated skin compared to non-treated skin. Increased tenascin expression was found in irradiated skin. The number of dermal blood vessels visualized by FVIII immunostaining was slightly higher in irradiated than in control skin. The amount of mast cells positive for tryptase, Kit receptor and chymase was increased in the upper dermis of irradiated skin. No difference in epidermal regeneration was found between irradiated and non-treated skin.

The results of this study suggest that alteration of collagen metabolism contributes to dermal side effects of therapeutic irradiation.

Keywords: breast neoplasms, collagen, radiotherapy/adverse effects, skin

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Oulu, August 2006

Riitta Rieki

Abbreviations

| | |
|---------------|-------------------------------------------------------------|
| CTGF | connective tissue growth factor |
| FGF | fibroblast growth factor |
| hnRNA | heterogeneous nuclear ribonucleic acid |
| ICTP | cross-linked carboxyterminal telopeptide of type I collagen |
| IFN- γ | Interferon gamma |
| IL | interleukin |
| MMP | matrix metalloproteinase |
| MT-MMP | membrane-type matrix metalloproteinase |
| PDGF | platelet derived growth factor |
| PINP | aminoterminal propeptide of type I procollagen |
| PIIINP | aminoterminal propeptide of type III procollagen |
| SBF | suction blister fluid |
| TEWL | transepidermal water loss |
| TGF- β | transforming growth factor beta |
| TNF- α | tumour necrosis factor alpha |
| TIMP | tissue inhibitor of matrix metalloproteinase |
| VEGF | vascular endothelial growth factor |

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I Riekkö R, Jukkola A, Sassi M-L, Höyhty M, Kallioinen M, Risteli J & Oikarinen A (2000) Modulation of skin collagen metabolism by irradiation: collagen synthesis is increased in irradiated human skin. *Br J Dermatol* 142: 874–880.
- II Sassi M-L, Jukkola A, Riekkö R, Höyhty M, Risteli L, Oikarinen A & Risteli J (2001) Type I collagen turnover and cross-linking are increased in irradiated skin of breast cancer patients. *Radiother Oncol* 58:317–323.
- III Riekkö R, Jukkola A, Oikarinen A & Kallioinen M (2001) Radiation therapy induces tenascin expression and angiogenesis in human skin. *Acta Derm Venereol* 81:329–333.
- IV Riekkö R, Parikka M, Jukkola A, Salo T, Risteli J & Oikarinen A (2002) Increased expression of collagen type I and III in human skin as a consequence of radiotherapy. *Arch Dermatol Res* 294:178–184.
- V Riekkö R, Harvima I, Jukkola A, Oikarinen A (2004) The production of collagen and the activity of mast cell chymase increase in human skin after irradiation therapy. *Experimental Derm* 13:364–371.

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1 Introduction

In the treatment of breast cancer, radiotherapy is used to reduce the local recurrence rates. Radiotherapy may induce both acute and late side effects. However, treatment alternatives for cancer are limited. The late side effects of radiation occur from a few months to several years after treatment. Fibrosis is a common late side effect of radiotherapy treatment for cancer. Fibrosis is characterized by a loss of flexibility and impaired function of the involved tissue. Other side effects of radiotherapy, such as neuropathy, pain and lymphoedema, are often associated with fibrosis. The pathogenetic mechanisms of radiation-induced fibrosis are not yet clear. In skin, the effects of radiation can be readily investigated.

Changes in collagen metabolism apparently have an important role in the formation of fibrosis. Collagens are the most abundant proteins of the extracellular matrix. They give tissues structural integrity and tensile strength. Over 20 distinct types of collagens have been characterized so far. Each collagen type has distinct tissue distribution, function and properties. In skin, collagen comprises 70–80% of its dry weight. The most abundant dermal proteins are type I and III collagens that are produced by fibroblasts. Type I collagen comprises 80–85% and type III collagen 10–15% of skin collagen. Type I and III collagens are synthesized as precursor molecules that have propeptides at both aminoterminal and carboxyterminal ends. These propeptides are cleaved off during the extracellular stages of collagen biosynthesis. Normally the balance between extracellular matrix protein synthesis and degradation is sustained by a complex system of cellular and cell-matrix interactions. Alterations in collagen synthesis are seen during ageing, in various pathological conditions, such as scleroderma, and in association with several medical therapies.

Matrix metalloproteinases (MMPs) are a group of enzymes capable of degrading virtually all extracellular matrix components. The MMPs have an essential role in collagen degradation. The proteolytic activity of MMPs can be inhibited by specific tissue inhibitors of matrix metalloproteinases, the TIMPs. The effects of irradiation of MMPs and their inhibitors, TIMPs, are not well documented.

While collagens provide tensile strength, the recoil properties of skin are largely due to elastin. Dermal elastin is synthesized by fibroblasts. In previous studies, elastic fibres in radiotherapy-treated human skin have not been analysed.

Tenascin is a large glycoprotein of the extracellular matrix. In most adult tissues its expression is restricted or absent. In skin, tenascin has been associated with wound healing and various malignant lesions. Tenascin expression in radiotherapy-treated human skin has not been previously studied.

Mast cells in the dermis may have a role in promoting fibrosis. Several lines of evidence suggest that mast cells increase fibroblast proliferation and collagen synthesis.

The purpose of the present study was to examine the effect of breast cancer radiotherapy on human skin collagen metabolism and on certain other components of skin extracellular matrix, such as elastic fibres and tenascin. The histological findings and physio-mechanical properties of radiotherapy-treated skin were compared with those of non-treated skin. Wound regeneration in radiotherapy-treated skin was studied using suction blister as a model. The possible role of mast cells in radiation-induced fibrosis was also studied.

2 Review of the literature

2.1 Structure and classification of collagens

Collagens are a family of proteins with certain structural and functional similarities, constituting major components of the extracellular matrix. In the human body, collagens are the most abundant proteins, amounting to an average of 3–4 kg in an adult. The primary structure of a collagen molecule polypeptide, the α -chain, includes repeated (Gly-X-Y) amino acid sequences. In this triplet, Gly represents glycine and X and Y almost any other amino acids, frequently proline or its hydroxylated forms. A collagen molecule consists of three polypeptide α -chains, each of which is coiled into a left-handed helix. The three helical α -chains are twisted around one another to form a right-handed triple-helical molecule. All three α -chains in a collagen molecule can be similar, or the molecule may consist of two or three different chain types. Collagen molecules form aggregates that have a supporting function in the extracellular matrix of tissues (Prockop & Kivirikko 1995, Oikarinen 1997).

Collagens can be divided into two subclasses, fibril-forming and non-fibril forming, according to their structural features. The fibril-forming collagens include type I, II, III, V and XI collagens. They are similar in size, contain large triple-helical domains with approximately 330 (Gly-X-Y) repeats per chain, are synthesized as large precursor molecules and assemble into fibrils and fibres. Type I collagen is found in most tissues, and it accounts for at least 70% of all collagens in the human body. There is about 2–3 kg of type I collagen in an adult, distributed mainly in the skeleton where it constitutes about 90% of the organic bone matrix. Type II collagen is a component of cartilage, cornea and vitreous humour. Type III collagen is distributed in various soft tissues. Type V collagen exists in minor amounts in tissues containing type I collagen, and type XI collagen is present in tissues containing type II collagen.

The non-fibril forming collagens can be further divided into subgroups: network-forming collagens, beaded filament-forming collagen, anchoring fibril-forming collagen, fibril-associated collagens with interrupted triple helices (FACIT), collagens with a transmembrane domain and proteins with multiple triple helix domains and interruptions (MULTIPLEXINs). The network-forming collagens include type IV collagen found in

basement membrane zone, type VIII collagen expressed in many tissues and type X collagen present in hypertrophic cartilage. Type VI collagen, present in most connective tissues, is the only known collagen that forms beaded filaments. Type VII collagen forms anchoring fibrils in epithelial tissues. FACIT collagens (types IX, XII, XIV, XVI, XIX, XX, XXI) are found in connection with the fibrils of the fibril-forming collagens in many tissues, linking them with other ECM molecules. The FACIT collagens have short triple-helical domains interrupted by short non-collagenous regions, and they do not form fibrils themselves. Transmembrane domain containing type XIII collagen is found in minor amounts in many tissues, and type XVII collagen of the same subgroup is distributed in the basement membrane zone. Type XV and XVIII collagens, the MULTIPLEXINs, are found in several tissues (Prockop & Kivirikko 1995, Koch *et al.* 2001, Fitzgerald & Bateman 2001).

There are also proteins with collagenous sequences, including the complement subcomponent C1q, the tail structure of acetylcholinesterase, pulmonary surfactant proteins and macrophage scavenger receptors. These proteins are not defined as collagens, since they do not fulfil the functional criteria of a collagen protein (Prockop & Kivirikko 1995).

2.2 Biosynthesis of collagen

2.2.1 Intracellular stages

Each of the different collagen types is encoded by specific genes, found in various chromosomes of the human genome (for review, see Prockop & Kivirikko 1995). The cells capable of collagen synthesis include fibroblasts, chondrocytes, odontoblasts, osteoblasts, endothelial cells and smooth muscle cells. In soft connective tissues such as skin, collagen is mainly produced by fibroblasts.

Collagen biosynthesis contains numerous intra- and extracellular stages (Fig. 1). After transcription of the collagen genes and processing of the hnRNA, the consequential messenger-RNA (mRNA) is transferred from the cell nucleus to the cytoplasm where collagen synthesis takes place in the ribosomes of the rough endoplasmic reticulum. The highest rate of collagen synthesis is observed during early development, after which there is a gradual decrease (Oikarinen 1997).

The collagen molecule is first synthesized in a precursor form, the procollagen, consisting of three pro- α polypeptide chains (Fig. 2). The pro- α -chains are modified in a number of enzymatic hydroxylation and glycosylation reactions before the formation of a procollagen molecule. These modifications include hydroxylation of certain proline residues in the Y-amino acid position by prolyl 4-hydroxylase to 4-hydroxyproline (also referred to as hydroxyproline), hydroxylation of some proline residues in the X-amino acid position to 3-hydroxyproline by prolyl 3-hydroxylase, and hydroxylation of some lysine residues to hydroxylysine by lysyl hydroxylase. The hydroxylation of proline residues to hydroxyproline is functionally the most important modification, since it is a

prerequisite for the later triple helix formation. The hydroxylation of certain lysines is necessary for the later formation of stabilizing cross-links in collagen fibres. Glucosyltransferases and galactosyltransferases add sugar residues to the hydroxylysyl groups. All these enzymes are specific for collagen biosynthesis.

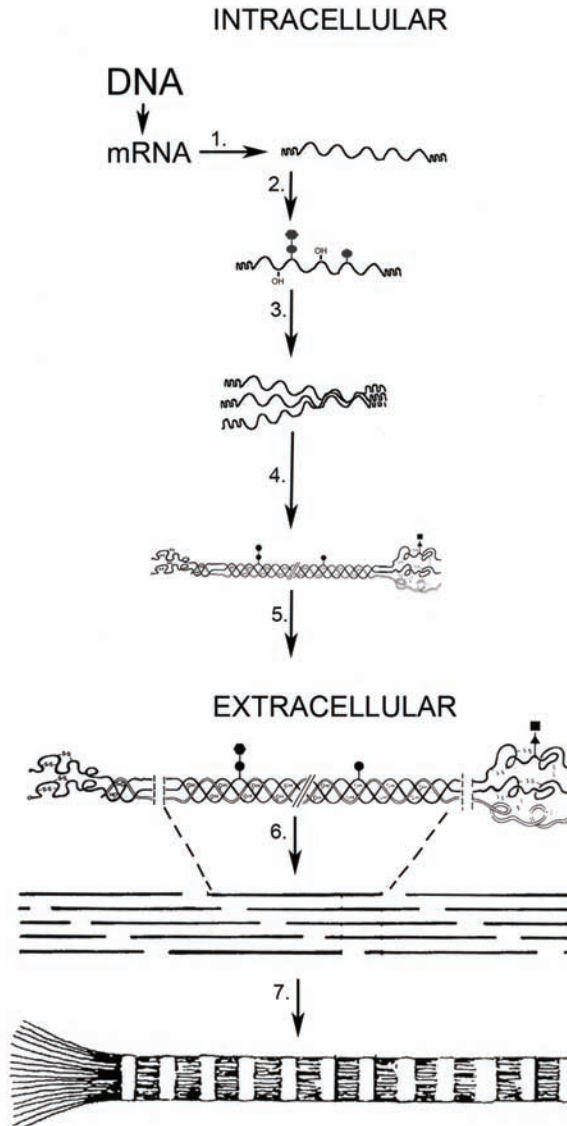


Fig. 1. Schematic representation of collagen biosynthesis. 1. Translation, processing of hRNA and synthesis of pro α -chains, 2. hydroxylation and glucosylation reactions, 3. alignment and self-assembly into procollagen triple-helices, 4.-5. secretion, 6. cleavage of procollagen propeptides, alignment and self-assembly of molecules into fibrils, 7. further aggregation of fibrils into collagen fibres and formation of cross-links (modified from L. Risteli).

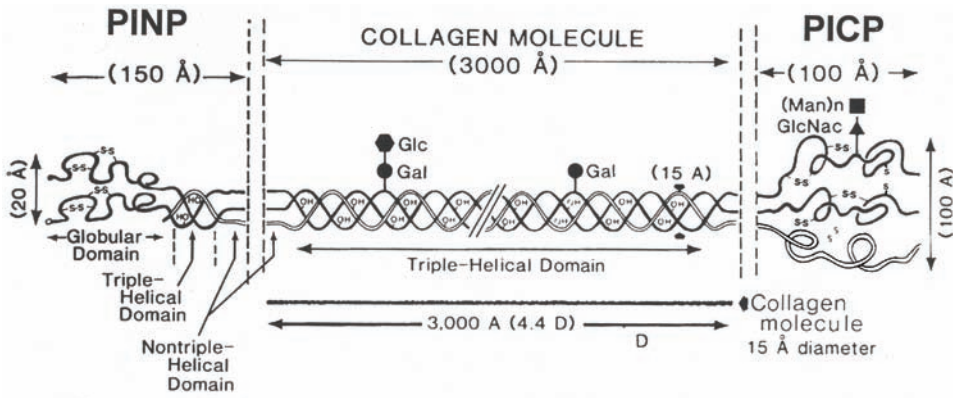


Fig. 2. Type 1 procollagen and collagen (modified from L. Risteli).

The individual pro- α -chains then become properly aligned in triplets to form a procollagen molecule and are linked by disulfide bonds at their carboxyterminal ends. As the pro- α -chains coil around each other to form a right-handed super-helical conformation, the enzymatic reactions stop. The presence of glycine in every third amino acid position of the repeat (Gly-X-Y) sequence enables the α -chains to coil into a triple-helical conformation. After that, the novel procollagen molecules are packed into vesicles in the Golgi apparatus and secreted towards the plasma membrane (Olsen 1991, Prockop & Kivirikko 1995, Oikarinen 1997).

2.2.2 Extracellular stages

After the procollagen molecules have been secreted into the extracellular space, they are further processed. At this stage of biosynthesis, the conformation of the procollagen molecule is triple-helical except for the large non-helical domains at its both amino- and carboxyterminal ends, the propeptides. The propeptides are removed *en bloc* by specific enzymes. C-proteinase cleaves off the carboxyterminal propeptides of type I, II and III procollagen molecules. For the cleavage of the aminoterminal propeptides there are two different enzymes: one N-proteinase for type I and II and another for type III procollagen. The amino- and carboxyterminal propeptides are also released in a 1:1 ratio to the collagen molecules.

After the procollagen propeptides have been removed, the novel collagen molecules assemble rapidly and spontaneously. This alignment of the collagen molecules is primarily determined by the distribution, charge and hydrophobicity of the amino acids in their α -chains. The collagen molecules first polymerize into fibrils. Each collagen molecule in a fibril is longitudinally staggered by about one quarter of its length relative to the adjacent molecule. The fibrils then further entwine to form collagen fibres (Kadler *et al.* 1996).

Within the nascent collagen fibres, advanced bonds are slowly formed between the adjacent molecules. The extracellular enzyme lysyl oxidase catalyses a reaction where certain lysine and hydroxylysine residues of a collagen polypeptide are converted into reactive aldehydes. This enables the bonding of two polypeptide chains with each other, i.e. the formation of bivalent cross-links. With time, more complicated cross-links connecting three or more polypeptide chains, i.e. trivalent and multivalent cross-links, are formed (Fig. 3). These bonds give the fibre tensile strength and stability. In mature collagen fibres, cross-links are mainly formed between the short non-triple-helical parts at both ends of the collagen molecule, the telopeptides, and certain areas within the triple-helical part of an adjacent collagen molecule (Eyre 1984, Olsen 1991, Oikarinen 1997). Two different enzymatic pathways have been described for the cross-linking of fibrillar collagens. The lysine-aldehyde pathway is predominant in adult skin, cornea and sclera. Generally, the hydroxylysine aldehyde pathway predominates in connective tissues that bear large mechanical loads, such as bone and cartilage (Eyre 1984).

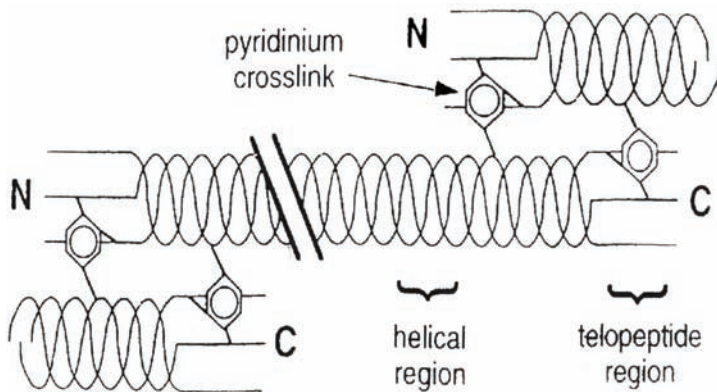


Fig. 3. The location of intermolecular cross-link within collagen fibril(modified from J. Risteli).

In skin, there is a specific cross-link, histidinohydroxylynonorleucine (HHL), within mature type I collagen fibres. The HHL-cross-link is trivalent and is located in the carboxyterminal telopeptide region of the collagen molecule. In addition, another trivalent cross-link called pyridinoline analogue (PA) or 3-deoxypyridinoline, has been suggested to exist in skin in the same molecular position. The amount of HHL-cross-linked type I collagen has been observed to increase during skin ageing (Yamauchi *et al.* 1988) and in the skin of systemic sclerosis patients (Ishikawa *et al.* 1998). The cross-linking of type III collagen has been suggested to occur via similar pathways as in type I collagen. Cross-linking of collagen molecules also occurs in non-enzymatic reactions with glucose (Eyre 1984).

2.3 Collagens in skin

2.3.1 Type I and III collagens

In skin, collagen is the major structural protein, constituting 70–80% of its dry weight. Presently, at least 12 distinct types of collagen have been detected in skin. The fibrillar type I and III collagens are the most abundant dermal collagens.

Type I collagen accounts for about 80% of collagen in adult human skin, providing essential tensile strength (Oikarinen 1997). A normal type I collagen molecule is composed of two identical $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain. In addition, suggested variants of type I collagen in humans include an $\alpha 1$ -homotrimer present in certain pathological conditions and the so-called onco-foetal collagen. Similarly to other fibril-forming collagens, type I collagen is synthesized as a precursor containing large extra polypeptide domains at both ends of the molecule. These domains, the aminoterminal propeptide of type I procollagen (PINP) and the carboxyterminal propeptide (PICP), are cleaved off by specific enzymes during the extracellular processing of the nascent collagen molecule. The functions of the propeptide domains are related to chain assembly and prevention of premature fibre formation. The molecular mass of PINP is 35,000, and it consists of three subunits: Col 1 (the globular, most aminoterminal region), Col 2 (the short, non-triple-helical domain adjacent to the collagen molecule) and Col 3 (the central, triple-helical domain). The pro $\alpha 2(1)$ chain lacks the Col 1 part. The Col 1 parts of the pro $\alpha 1(I)$ chains are the most immunogenic parts of the PINP molecule (Fig.2). (Risteli *et al.* 1995) The PINP molecule released from tissues is transported in blood circulation or via lymphatic vessels into the liver, where it is eliminated through scavenger receptors of endothelial cells (Melkko *et al.* 1994). The PICP molecule is cleared by mannose receptors of liver endothelial cells, which recognize the specific oligosaccharide chains of PICP molecules (Smedsrød *et al.* 1990).

The genes encoding the $\alpha 1(I)$ and $\alpha 2(I)$ chains of type I collagen are located on human chromosomes 17 and 7, respectively (Prockop & Kivirikko 1995). Although located apart, the expressions of these two genes are coordinately regulated. Several factors have been observed to modify type I collagen synthesis, including cell-matrix interactions and cytokine-mediated cellular interactions. The synthesis of both type I collagen chains is regulated by different cytokines at the transcriptional level (for review, see Rossert & Garret 1995). Regulatory regions within the promoters of both type I collagen subunits have been analysed, and observed to contain several repressor and enhancer elements. These include cytokine-responsive elements, like TGF- β response elements, TNF- α response element and IFN- γ response element (Ghosh 2002). Different transcription factors interacting with collagen genes have also been characterized (for review, see Ghosh 2002). TGF- β has been observed to act as chemoattractant to fibroblasts, to stimulate the synthesis and secretion of ECM proteins including type I and III collagens and to decrease ECM degradation (Rossert & Garret 1995). TNF- α has been observed to decrease the transcription of type I collagen *in vitro*, but in serum-free conditions it has also been shown to increase collagen production by fibroblasts (Rossert & Garret 1995). IFN- γ has been shown to decrease cutaneous fibrosis after wounding and to decrease

fibroblast proliferation and type I collagen synthesis (Rossert & Garret 1995). Glucocorticoids have been shown to decrease both collagen synthesis and the corresponding collagen mRNA level in human skin (Oikarinen *et al.* 1998).

Type III collagen is the second most common of collagens. It is found in soft tissues, including skin, together with type I collagen. In skin, type III collagen accounts for about 15% of collagen and provides important additional tensile properties to the tissue (Oikarinen 1997). Type III collagen is expressed particularly in young and metabolically active connective tissue, such as healing wounds (Oikarinen 1997). A type III collagen molecule is composed of three identical 1(III) chains. Similarly to type I collagen and other fibrillar collagens, type III collagen is also synthesized as a precursor molecule with propeptide domains attached to both its aminoterminal (PIIINP) and carboxyterminal (PIIICP) ends. During the extracellular processing of the developing collagen molecule, the PIIINP and PIIICP molecules are cleaved off by specific proteinases. The PIIINP molecule has a molecular weight of 42,000, and it consists of three subdomains: Col 1 (the most aminoterminal, globular domain), Col 2 (a non-collagenous, most carboxy-terminal domain), and Col 3 (the central, triple-helical domain). The aminoterminal domain Col 1 is the most immunogenic region of the molecule (Rohde *et al.* 1983, Risteli 1993). Intact PIIINP molecule is eliminated through the scavenger receptor of liver endothelial cells (Melkko *et al.* 1994). Fewer details are available about the structure and biological role of PIIICP, since the molecule has not been purified in sufficient amounts (Burchardt *et al.* 1998). The gene for type III collagen is located on chromosome 2 (Prockop & Kivirikko 1995).

It has been shown that type I and type III collagens coexist within individual fibrils, and it has been suggested that these interactions may regulate collagen fibril formation and fibril growth (Fleischmajer *et al.* 1990).

2.3.2 Other collagens in skin

Other collagen types are found in skin in considerably lesser amounts than type I and III collagens. In the skin basement membrane zone, collagen types IV, VII and XVII are present. Type IV collagen is a major component of the lamina densa. Type VII collagen is found in anchoring fibrils, linking basement membranes to anchoring plaques of type IV collagen and laminin in the underlying ECM, while type XVII collagen is primarily located in the hemidesmosomes. Type V collagen is associated with type I/III copolymers in dermis. Type VI collagen is located in microfibrils of dermal connective tissue. Type VIII collagen is found in dermal vascular endothelium. Type XII and XIV collagens are distributed in the dermis, attached to the surfaces of the fibril-forming collagens. Type XIII collagen has been localized to the dermal-epidermal junction and to the periphery of keratinocytes, indicating a possible role as a cell adhesion molecule. Type XV collagen is found in the epidermis as a transmembrane collagen (Prockop & Kivirikko 1995, Peltonen *et al.* 1999, Uitto & Kouba 2000).

2.3.3 Collagen degradation

Controlled degradation of extracellular matrix proteins is essential to several physiological processes including development, tissue repair and angiogenesis. The collagen fibres are very resistant to proteolysis due to the triple-helical structure of the collagen molecules and the cross-links within the collagen fibres. Consequently, specific enzymes exist in the tissues for the degradation of collagens. Under physiologic conditions, the collagen turnover is rather slow (Risteli 1993).

Matrix metalloproteinases (MMPs) are a genetically, structurally and functionally related group of zinc-dependent endopeptidase enzymes capable of degrading virtually all extracellular matrix components. The MMPs hence have an essential role in collagen degradation. In skin, several cell types are capable of producing MMPs. These include keratinocytes, fibroblasts, macrophages, endothelial cells, mast cells, eosinophils and neutrophils (Kähäri & Saarialho-Kere 1997). Presently, at least 25 vertebrate MMPs and over 20 human homologues have been identified, each having distinct but often overlapping substrate specificities (Sternlicht & Werb 2001).

The general structure of most of the MMPs consists of a signal peptide, a propeptide, a catalytic domain and a haemopexin-like domain. The aminoterminal signal sequence of the MMPs is removed after it directs their synthesis to the endoplasmic reticulum. The propeptide domain maintains enzyme latency until it is removed or disrupted. The catalytic domain contains a zinc-binding region and dictates cleavage site specificity. The haemopexin domain influences binding of certain substrates and inhibitors, membrane activation, and some proteolytic activities (Kähäri & Saarialho-Kere 1999, Sternlicht & Werb 2001). The MMP enzyme family can be divided into several distinct subgroups based on preferential substrates or similar structural domains. These include collagenases, gelatinases, stromelysins, stromelysin-like MMPs, membrane-type MMPs (MT-MMPs) and novel MMPs (Kähäri & Saarialho-Kere 1999).

The collagenase subgroup includes MMP-1 (collagenase 1, fibroblast collagenase), MMP-8 (collagenase 2, neutrophil collagenase) and MMP-13 (collagenase-3). These proteinases are capable of initiating the degradation of native fibrillar collagen types I, II, III and V. The collagenases cleave fibrillar collagens at a distinct site to aminoterminal $\frac{3}{4}$ and carboxyterminal $\frac{1}{4}$ fragments, which denature to gelatin at body temperature and are further degraded by another subgroup of MMPs, the gelatinases. MMP-13 is distinguished by its ability to degrade type IV, IX, X and XIV collagens, tenascin-C and fibronectin in addition to fibrillar collagens and gelatin (Kähäri & Saarialho-Kere 1999).

The gelatinase subgroup contains MMP-2 (gelatinase-A, 72-kDa gelatinase) and MMP-9 (gelatinase-B, 92-kDa gelatinase). MMP-2 is expressed by various cell types, including keratinocytes and fibroblasts, whereas MMP-9 is produced by epithelial cells including keratinocytes. The gelatinases are apparently important in the final degradation of fibrillar collagens. (Kähäri & Saarialho-Kere 1999) MMP-2 is also able to cleave native type I collagen into similar fragments as the collagenases (Aimes & Quigley 1995). In addition, MMP-9 has been observed to cleave type I, II and V collagens in the aminoterminal non-helical telopeptide (Okada *et al.* 1995).

The stromelysins and stromelysin-like MMPs include MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-10 (stromelysin-2), MMP-11 (stromelysin-3) and MMP-12

(metalloelastase). This subgroup of MMPs has a wide range of ECM substrates including certain collagens, but not native type I and III collagens (Kähäri & Saarialho-Kere 1997).

The membrane-type MMPs (MT-MMPs) have a transmembrane domain at the carboxyterminal end. The subgroup includes MMP-14 (MT1-MMP), MMP-15 (MT2-MMP), MMP-16 (MT3-MMP) and MMP-17 (MT4-MMP). MMP-14 has been shown to degrade various ECM components, including native type I collagen and gelatin, and MMP-16 is capable of degrading type III collagen and gelatin. The structure and substrate specificities of the newer MMPs remain to be characterized (Kähäri & Saarialho-Kere 1999).

In addition to MMPs, there are other enzymes capable of degrading various ECM components, including collagens. Based on their catalytic mechanism and inhibitor sensitivities, these enzymes can be classified as serine proteinases (Cirino *et al.* 2000), cysteine proteinases (Chapman *et al.* 1997) and aspartic proteinases (Hoegl *et al.* 1999).

In general, the expression of MMPs is tightly regulated. At transcriptional level, MMPs can be induced by various factors, including cytokines, growth factors, altered cell-cell and cell-matrix interactions. The MMPs are also regulated at the level of activation of their latent precursors (zymogens). Activation of the latent MMPs can be accomplished by other MMPs or several other proteases in the extracellular space. The proteolytic activity of MMPs can be inhibited specifically by tissue inhibitors of matrix metalloproteinases (TIMPs). The TIMPs are a family of at least four proteins, TIMP-1, -2, -3 and -4, which reversibly bind to active MMPs in a 1:1 stoichiometric fashion. The TIMPs are produced by various cell types, including fibroblasts, keratinocytes, endothelial cells and osteoblasts. The individual TIMPs differ in their ability to inhibit various MMPs. For instance, TIMP-1 and TIMP-2 inhibit the activity of most MMPs. TIMP-1 is significantly more effective against MMP-1 than other MMPs, and TIMP-2 is an effective inhibitor of gelatinases. TIMP-1 and TIMP-2 are known to form complexes with latent MMP-9 and MMP-2, respectively. TIMP-3 and TIMP-4 have also been observed to inhibit several distinct MMPs. In addition to TIMPs, MMP activity can be inhibited by non-specific inhibitors, including the 2-macroglobulin and 1-antiprotease. (Kähäri & Saarialho-Kere 1999, Sternlicht & Werb 2001)

In addition to extracellular collagen degradation by proteolytic enzymes such as MMPs, collagen can also be degraded intracellularly. Intracellular degradation has been observed particularly when the newly synthesized collagen is abnormal (Bateman *et al.* 1984). Collagen fibril fragments may also be digested within the lysosomal apparatus after phagocytosis. Phagocytosis and intracellular collagen degradation have been suggested to occur in both pathological and physiological situations, but the mechanisms remain to be elucidated (Everts *et al.* 1996).

As a final result of collagen digestion, small peptides and amino acids are formed. The digested material can be phagocytosed by macrophages and fibroblasts. Some of the degradation products are liberated in the blood circulation, where they are taken up and metabolized by the liver. Part of the degraded material is excreted in the urine (Risteli 1993).

2.3.4 Methods to study collagen metabolism in skin

There are several methods available for analysing skin collagen metabolism. Skin hydroxyproline content can be used to determine the total collagen amount in skin. In this method, skin biopsy samples are hydrolysed and the hydroxyproline content is measured chemically (Kivirikko *et al.* 1967).

Skin collagen content can be analysed with a method based on selective binding of Sirius red F3BA and Fast green FCF to collagen and non-collagenous components. This method utilizes formalin-fixed paraffin-embedded biopsy samples (Lopez-De Leon & Rojkind 1985). Other staining methods to visualize dermal collagen include Haematoxylin-eosin, Masson's trichrome and van Gieson stains.

The rate of collagen synthesis can be studied by measuring the activity of certain enzymes. The activities of prolyl hydroxylase and glucosyltransferase can be measured from skin biopsies (Kuutti-Savolainen & Kero 1979). The collagen synthesis rate can also be analysed by incubating skin samples with radioactive proline and determining the formation of radioactive hydroxyproline (Uitto 1970).

It is possible to analyse collagen synthesis by isolating collagen messenger-RNA (mRNA) and measuring it with specific probes (Moy *et al.* 1988). This method estimates the total mRNA amount in the sample. Relatively large skin specimens are required, and mRNA is easily degraded. A quantitative polymerase chain reaction method requiring a lesser amount of total RNA is also available (Tasanen *et al.* 1996).

By *in situ* hybridization, the cells with active collagen synthesis can be localized in skin samples using specific RNA probes. Paraffin-embedded tissue sections are used in this method. Both radioactive and non-radioactive *in situ* hybridization techniques exist (Mäkelä *et al.* 1999). *In situ* hybridization cannot be used for a direct quantitative analysis.

Skin collagen synthesis *in vivo* can be analysed by measuring the level of procollagen propeptides in suction blister fluid (SBF) with a specific radioimmunoassay (Oikarinen *et al.* 1992, Risteli *et al.* 1995). This method is non-invasive and practically painless for the subject. Procollagen propeptides can also be determined by radioimmunoassay from wound fluids and serum (Haukipuro 1991, Risteli & Risteli 1995).

Cell cultures are also used to study collagen metabolism. This method is useful for isolation and characterization of collagen polypeptides, collagen mRNA and DNA. It must be considered, however, that the complex interactions of the extracellular matrix *in vivo* can only partly be characterized *in vitro*.

Skin collagen degradation can be studied by localizing the degenerated fibrils in histological samples. Electron microscopy can also be utilized. Indirect evidence of collagen degradation can be acquired by using antibodies against various cytokines that are known to be associated with collagen degradation. The levels of collagen-degrading enzymes can also be measured either by assaying their specific mRNAs in skin samples or by analysing suction blister fluids (Annala *et al.* 1993), or from tissue slides (Fisher *et al.* 2001).

2.4 Other proteins of dermal extracellular matrix

2.4.1 Elastin

Elastic fibres are present in connective tissues of several organs, such as blood vessels, lungs, ligaments, tendons and skin. In skin, elastic fibres constitute a relatively small proportion, 2-4 %, of skin dry weight. Despite their comparatively low quantity, they are responsible for the resilient and elastic properties of skin. The protein elastin is the main constituent of the elastic fibres present in the dermal extracellular matrix, accounting for over 90% of their mass. (Rosenbloom *et al.* 1993, Vrhovski & Weiss 1998)

The elastin gene has been isolated and localized to chromosome 7 in humans. It has been observed that elastin gene expression initiates during early embryonic development and remains relatively constant for several decades, decreasing in senescence (Fazio *et al.* 1988, Fazio *et al.* 1991). Expression of elastin precursor mRNA and elastic fibre synthesis are proposed to be highest in early development and in rapidly growing tissues (Rosenbloom *et al.* 1993, Vrhovski & Weiss 1998).

Dermal elastin is biosynthesized by fibroblasts as a precursor molecule, tropoelastin. Tropoelastin polypeptides are composed of two types of segments: hydrophobic segments accountable for the elastic properties of the molecule, and alanine- and lysine-rich segments that participate in the formation of cross-links between adjacent molecules (Rosenbloom *et al.* 1993).

After translation, the soluble tropoelastin molecules are secreted from the Golgi apparatus into the extracellular space. Deposition of tropoelastin occurs at specific regions of the cell surface. The newly deposited tropoelastin molecules are thought to aggregate in a process of coacervation, which is considered to be an important step in fibrillogenesis. Subsequently, covalent cross-link bindings are formed between the polypeptide chains. The cross-link formation is catalysed by the lysyl oxidase enzyme. Consequently, the tropoelastin molecules become insoluble. Cross-linking is essential for the normal physiological function of elastin (Brown-Augsburger *et al.* 1995, Vrhovski & Weiss 1998).

The mechanisms of elastin fibrillogenesis are not known in great detail. As the elastic fibre develops, elastin forms a central core that is covered by microfibrils secreted to the cell surface. These microfibrils consist of a number of glycoproteins, such as fibrillin. Fibrillin is apparently crucial for the normal function of the elastic fibres. It has been suggested that microfibrils serve as a scaffold to nascent elastic fibres on the cell surface. Several other components have also been suggested to be associated with elastic fibres and microfibrils, but the biological roles of many of these molecules still remain to be characterized (Rosenbloom *et al.* 1993, Brown-Augsburger *et al.* 1995, Vrhovski & Weiss 1998, Kielty *et al.* 2002).

A mature elastic fibre is also composed of a highly insoluble elastin protein core sheathed by microfibrils. The elastic fibres intertwine among the collagenous fibres of dermal connective tissue to form an extensive, thin network. In the lower dermis the fibres appear larger in diameter than in the upper dermis. Several structural models have been presented in order to explain the function and recoil properties of elastin; however,

these qualities of elastin have not yet been decisively characterized (Vrhovski & Weiss 1998).

The turnover of cross-linked, insoluble elastin is apparently very slow under physiological conditions. It has been considered that elastin may last for the entire lifetime of the host. The main group of proteases capable of degrading elastin are the elastases (Vrhovski & Weiss 1998, DeBelle & Tamburro 1999).

Dermal elastic fibres have been found to be affected during intrinsic ageing and also by photodamage, or photoageing. During the intrinsic ageing process, the rate of elastin gene expression is markedly reduced, leading to loss of elastic fibres, clinical skin atrophy and loss of recoil. Skin photodamage has been demonstrated to cause a massive accumulation of abnormal elastotic material in dermis, solar elastosis (Uitto & Bernstein 1998). In addition, elastic fibre abnormalities have been observed in several disorders affecting skin and other tissues, such as the inherited disorders pseudoxanthoma elasticum, cutis laxa and Menkes disease, Marfan syndrome caused by fibrillin gene mutations and acquired pathological conditions such as anetoderma, atherosclerosis and emphysema (Oikarinen *et al.* 1984, Vrhovski & Weiss 1998).

2.4.2 *Tenascin*

Tenascins are a family of extracellular matrix glycoproteins with similar multiple domains. Tenascin-C was the first known member of the group, discovered in chicken extracellular matrix (Chiquet & Fambrough 1984). Several related types of tenascin have since been described. These include tenascin-R, tenascin-X and tenascin-Y. In addition, a novel tenascin-W has been isolated (Jones & Jones 2000).

Tenascin-C (also referred to as tenascin or cytotactin) is a complex of six structurally similar polypeptide chains. Each of these polypeptide chains is composed of several types of repeated sequences, such as epidermal growth factor-like repeats and fibronectin type III domains. The distal part of the polypeptide chain is composed of a globular domain resembling fibrinogen. The polypeptide chains of tenascin-C are linked at their aminoterminal part by a tenascin assembly domain to form a six-armed symmetrical structure. The other known tenascin proteins have similar linear arrangement of protein domains, but are not known to form hexamers (Jones & Jones 2000).

The distribution of Tenascin-C varies remarkably in different physiological and pathological conditions. Tenascin-C is prominently synthesized during various stages of embryogenesis (Chiquet-Ehrismann 1995). The expression of tenascin in adult tissues is rather limited. In normal human skin tenascin is primarily localized in the papillary dermis, and is also found in association with cutaneous blood vessels and adnexae (Lightner *et al.* 1989, Koukoulis *et al.* 1991). An increase of tenascin-C expression has been observed during wound healing, in hyperproliferative skin diseases, in scleroderma and several tumours including melanomas and most carcinoma types (Mackie *et al.* 1988, Koukoulis *et al.* 1991, Schalkwijk *et al.* 1991, Lacour *et al.* 1992, Chiquet-Ehrismann 1995, Latijnhouwers *et al.* 1997). An upregulation of Tenascin-C has been observed in pig skin after high-dose gamma radiation (Geffrotin *et al.* 1998). The structure of the gene

encoding human tenascin-C has been determined, and the gene has been localized to chromosome 9 (Gulcher *et al.* 1991).

Tenascin-R expression is apparently restricted to the central nervous system (Erickson 1993, Carnemolla *et al.* 1996). Using animal models, tenascin-X has been detected in several tissues including skin, tendon, muscle, and blood vessels. In normal human skin, tenascin-X expression has been observed throughout the dermis. Tenascin-X deficiency has been found to cause a distinct form of Ehlers-Danlos syndrome (Schalkwijk *et al.* 2001). The genes of human tenascin-R and tenascin-X have been localized to chromosomes 1 and 6, respectively (Carnemolla *et al.* 1996, Bristow J *et al.* 1993). Tenascin-Y has been detected in chicken connective tissue and skin (Hagios *et al.* 1999). Tenascin-W was discovered in zebrafish, and has to date not been identified in mammal tissues (Weber *et al.* 1998).

Cultured human dermal fibroblasts and epidermal keratinocytes have been shown to secrete tenascin-C (Latijnhouwers *et al.* 1997). In addition, various cell types of several other tissues have been observed to produce tenascin-C (Chiquet-Ehrismann 1995). Tenascin-X is known to be secreted by human dermal fibroblasts *in vitro* (Schalkwijk *et al.* 2001).

Tenascin synthesis can be upregulated *in vitro* by several growth factors and cytokines, such as TGF- β , fibroblast growth factor-2, TNF- α , IL-1 and IL-4. In addition, vasoactive peptides, other extracellular matrix proteins and biomechanical factors have been proposed to modulate expression of tenascins (Chiquet-Ehrismann 1995, Jones & Jones 2000).

Proposed cell surface receptors for the tenascins include members of the integrin family, cell adhesion molecules of the immunoglobulin superfamily, a transmembrane receptor protein phosphacan, and annexin II (Jones & Jones 2000).

Various functions for tenascin have been suggested, but its exact role *in vivo* remains to be defined. It has been proposed that the functions of the different tenascin proteins are partly overlapping. In cell cultures, tenascin-C has been observed to have both adhesive and anti-adhesive activities, suggesting a role in cellular migration *in vivo* (Erickson 1993). Tenascin-C has also been found to be able to both stimulate and inhibit cell division. In animal models, tenascin-C knockout mice have been born alive but show several defects, such as abnormal behaviour, abnormal brain chemistry and abnormally low fibronectin expression in healing skin wounds (Mackie & Tucker 1999). The distribution of tenascins described in several immunohistochemical and *in situ* hybridization studies suggests a role in developmental processes and tissue remodelling.

2.5 Effect of therapeutic irradiation on skin

2.5.1 Therapeutic irradiation

In general, ionizing radiation is defined as radiation that has sufficient energy to eject one or more electrons from an atom or a molecule. As a result, energy is released. The release

of energy caused by ionizing radiation passing through a tissue causes damage on a cellular level, resulting in cell and tissue death. Radiation may cause cellular injury either directly by damaging DNA, or indirectly via the production of free radicals, leading to inhibition or failure of mitosis. Cellular radiosensitivity varies depending on the cell cycle phase. In general, cells are most radiosensitive when in the premitotic or mitotic phase, and most radioresistant when in the synthetic phase. Several different types of ionizing radiation may be used for therapeutic purposes, including particulate radiation and electromagnetic radiation. Particulate radiation consists of electrons, protons, alpha particles, and neutrons. Gamma rays and x-rays are electromagnetic radiation, composed of photons (Tokarek *et al.* 1994).

There is a difference between the response of normal cells and tumour cells to radiation. Normal cells are more capable of repairing themselves following a limited amount of radiation than malignant cells. This greater reparative capability of normal cells over tumour cells is utilized by administering radiation treatment in multiple small doses, fractions, over a period of time, usually weeks. Thus, preferential destruction of malignant cells can be achieved by using therapeutic irradiation. The unit of absorbed radiation dose is the gray (Gy). Several different methods for delivering therapeutic radiation exist (Tokarek *et al.* 1994).

2.5.2 Acute phase normal tissue reactions to radiotherapy

Normal tissue response to ionizing radiation involves cell death, the production of free radicals, alterations in gene expression and production of cytokines. In addition, cell-matrix interactions alter the extracellular component of the tissue (Travis 2001). The factors contributing to radiation reaction include radiation dose, type and energy of radiation, treatment time and volume of radiation field (Tokarek *et al.* 1994). In the skin, acute radiation reactions occur frequently because of the relatively high rate of mitosis in the epidermis. Other tissues sensitive to acute reactions due to their high mitotic rate include bone marrow and oral and gut mucosa.

Acute skin reaction is the most frequently occurring side effect of therapeutic irradiation. It has been estimated that 90–95% of patients undergoing radiation therapy experience some degree of acute skin reaction (Porock *et al.* 1999). In general, skin reactions occurring in less than six months after radiotherapy are termed acute (Tokarek *et al.* 1994).

The first clinical manifestation of acute skin radiation reaction is erythema, which during the first few post-irradiation days is thought to represent early injury to dermal capillaries, leading to dilatation and leakage of superficial dermal vessels. Oedema and endothelial proliferation in the vascular walls may occur. In the following weeks, an increase of erythema can be observed as a result of basal keratinocyte destruction and consequent inflammatory reaction. Another clinical effect of radiation on skin and particularly on keratinocytes is dry desquamation, as surviving keratinocytes migrate to repopulate the epidermis during the next few weeks. As a result of extensive destruction of basal cells by radiation, varying degrees of epidermal loss termed moist desquamation can be observed. In dermal tissue, acute radiation effects may include depletion of

parenchymal cells, such as fibroblasts. On rare occasions after very intense radiation damage, bulla formation, ulceration or tissue necrosis may develop as a result of epidermal and dermal destruction. In addition, alopecia in the hair-bearing areas may develop as a result of radiation damage to adnexal structures (Tokarek *et al.* 1994, Elder *et al.* 1997, Porock *et al.* 1999).

2.5.3 Late phase normal tissue reactions to radiotherapy

Late radiation reactions generally occur from six months to several years after radiotherapy. The late radiation skin effects include pigmentary changes, fibrosis of skin and subcutaneous tissues, telangiectasia, and sebaceous and eccrine gland destruction. In addition, tissue necrosis and secondary tumours may evolve (Tokarek *et al.* 1994).

Late radiation injury in keratinocytes may result in hyperkeratosis or atrophy (Elder *et al.* 1997). As melanocytes are exposed to radiation, an increased production and transfer of melanin pigment is frequently observed. Melanophages may also engulf dermal pigment, resulting in clinical pigmentary changes as a result of irradiation. As a result of melanocyte destruction after higher doses of radiation, depigmentation may also occur (Tokarek *et al.* 1994).

As a late vascular radiation change, occlusion, oedema and fibrous thickening of the vascular walls, thrombosis and recanalization of existing vessels have been observed. In the upper dermis, telangiectatic vessels may be found. Lymphoedema in the superficial dermis may also occur (Tokarek *et al.* 1994, Elder *et al.* 1997).

2.5.4 Effect of ionizing radiation on fibroblasts and collagen metabolism

Fibrosis is a complex tissue response characterized by excessive deposition of ECM components, particularly collagens (Trojanowska *et al.* 1998). Fibrosis may develop as a result of inflammation or tissue injury, including exposure to ionizing radiation. It has been postulated that there are similarities between the mechanisms of physiological tissue repair and fibrogenesis, and that fibrogenesis represents an uncontrolled and abnormal process of wound healing (Border & Noble 1994, Trojanowska *et al.* 1998, Travis 2001).

A modulation of collagen synthesis has been observed as a result of irradiation (Barcellos-Hoff 1993, Autio *et al.* 1998), but the mechanisms leading to fibrogenesis are not known in detail. It has been suggested that ionizing radiation induces in mitotically active fibroblast progenitor cells a premature differentiation into post-mitotic fibrocytes capable of the synthesis of interstitial collagens (Rodemann & Bamberg 1995).

Several lines of evidence suggest that transforming growth factor beta (TGF- β) is a central factor in fibrogenesis. TGF- β is a multifunctional cytokine, consisting of a family of three mammalian isoforms, TGF- β 1, TGF- β 2 and TGF- β 3. TGF- β has been shown to have roles in regulating repair and regeneration following tissue injury, including inflammation and angiogenesis (Border & Ruoslahti 1992). TGF- β has been found to stimulate the synthesis of individual ECM components including fibronectin, tenascin,

collagens and proteoglycans, and to simultaneously inhibit matrix degradation by decreasing the synthesis of proteases and increasing the levels of protease inhibitors (Border & Ruoslahti 1992). It has been proposed that TGF- β is also a key cytokine in radiation-induced fibrosis, and it has been demonstrated that TGF- β can be induced by irradiation (Barcellos-Hoff 1993, Martin *et al.* 1993).

It has also been observed that as a result of irradiation, growth factors for fibroblasts are secreted by mononuclear phagocytes, including platelet-derived growth factor (PDGF), TNF- α and insulin-like growth factor (Thornton *et al.* 1996).

Remarkable inter-patient variability in radiation morbidity has been reported in several studies, and attempts have been made to predict individual radiation sensitivity by using fibroblast cultures, but the results have been controversial (Johansen *et al.* 1996, Peacock *et al.* 2000). In an animal model using pig skin, a prolonged lifespan of irradiated fibroblasts has been observed *in vitro* (Martin *et al.* 1989).

The data concerning the effect of irradiation on MMPs and their inhibitors, TIMPs, are scarce. It has been observed that ionizing radiation enhances MMP-2 production in human lung epithelial cell lines *in vitro*, whereas the expression of MMP-9 and TIMP-2 remains unaffected (Araya *et al.* 2001).

2.5.5 Fibrotic skin changes in radiation-treated breast cancer patients

Breast cancer is the most common type of cancer in women in the western countries. In Finland, over 3,500 new cases are diagnosed yearly. Breast cancer rarely develops before the age of 30 years and is more frequent in the age group of over 45 years. Although certain risk factors have been observed, the cause of breast cancer is presently obscure. In a minority of cases, the cause of breast cancer is genetic. In Finland, the five-year breast cancer survival rate is presently about 80% (Holli *et al.* 2002).

The treatment options of breast cancer include surgical removal of the tumour, radiotherapy and systemic therapy using either hormonal or cytostatic medications. Postoperational radiotherapy has been observed to significantly reduce local recurrences. In Finland, breast cancer radiotherapy is presently generally administered as a total dose of 50 Gy in 25–26 fractions. In addition, a booster to the operation scar may be administered. For early stage breast cancer, breast-conservation surgery and radiotherapy has become the standard treatment option (Holli *et al.* 2002).

Radiation-induced fibrotic lesions have been described in many tissues, including skin, lung, heart and liver (Rodemann & Bamberg 1995). Fibrosis is a serious complication in internal organs, but fibrosis may also limit the range of motion and impair function considerably in skin, subcutis and muscle as well. Fibrosis of the skin and breast is a common late complication of radiotherapy. Among breast cancer patient groups with variable radiotherapy protocols and follow-up times, fibrosis has been described in 29–86% of patients (Johansson *et al.* 2000, Meric *et al.* 2002). Fehlaue *et al.* observed definite increased density or very marked density, retraction and fixation of the irradiated breast area in 18–51% of the patient groups examined (Fehlaue *et al.* 2003). Breast fibrosis has been observed to be more frequent in patients with larger tumours and in

patients who received a boost (Meric *et al.* 2002). The use of large daily radiation fractions also contributes to the fibrotic conditions.

In breast cancer patients with a connective tissue disease such as rheumatoid arthritis, scleroderma, lupus erythematosus or mixed connective tissue disease, several cases of radiation fibrosis have been reported (Robertson *et al.* 1991, Mayr *et al.* 1997). Postirradiation morphea has also been described as a consequence of radiotherapy (Schaffer *et al.* 2000).

2.6 Other disorders characterized by fibrosis

Tissue fibrosis is a major pathological feature of several clinical disorders. The pathogenesis of these conditions is not known in detail. Scleroderma is a fibrotic disease involving skin and internal organs, and is often regarded as an example of a fibrotic process. In scleroderma, an excessive accumulation of dermal collagen is observed. It has been shown that the synthesis of type I and III collagens is equally increased in scleroderma fibroblasts *in vitro*. The activities of prolyl and lysyl hydroxylases have also been demonstrated to increase in scleroderma fibroblasts (Kähäri 1993).

TGF- β and CTGF are cytokines implicated in the pathogenesis of various fibrotic conditions. It has been suggested that TGF- β induces fibrosis in the early stages of systemic scleroderma, and then CTGF acts to maintain tissue fibrosis (Takehara 2000).

In addition to cytokines, various cell-cell and cell-matrix interactions have been proposed to have a role in fibrosis. Integrins are a family of proteins that mediate the interaction of skin fibroblasts with the surrounding extracellular matrix. They act as cell surface receptors, and are able to activate pathways of intracellular signalling. An increased expression of integrins has been observed in scleroderma, indicating enhanced cell-matrix interactions by scleroderma fibroblasts (Kähäri 1993).

Keloids are hypertrophic cicatricial lesions characterized by excessive dermal collagen deposition and often by a marked fibroblastic cellular infiltrate. It has been suggested that the pathogenesis of keloids and other cutaneous fibroses such as scleroderma may be closely related, and that the overproduction of TGF- β might be one of the major causes of hypertrophic scar and keloid formation (Zhou *et al.* 1997, Mori *et al.* 2002). It has been observed that in keloids the type I/III procollagen mRNA ratio is markedly increased, and the expression of type I procollagen genes thus appears to be selectively enhanced in keloids (Uitto & Kouba 2000).

2.6.1 Mast cells and fibrosis

Mast cells are found in dermis of normal skin, localized mainly around blood vessels, nerves and dermal appendages. Several lines of evidence suggest that mast cells may have a role in the pathogenesis of fibrosis. An abundance of mast cells has been found in scleroderma. Mast cells have also been suggested to contribute to wound healing and angiogenesis (Trabucchi *et al.* 1988, Irani *et al.* 1992, Levi-Schaeffer & Pe'er 2001).

Mast cells produce several pharmacologically active mediators, including histamine, heparin, serine proteases tryptase, chymase and cathepsin G-like proteinase and different growth factors and cytokines. Human mast cells can be divided into two major subclasses according to the constituents of their granules. The main subclasses of human mast cells are those containing tryptase (MC_T cells) and those containing tryptase, chymase, carboxypeptidase and a cathepsin-G like proteinase (MC_{TC} cells). The mast cells in normal human skin are predominantly of the latter type (Huttunen 2000).

Several mast cell cytokines, such as TGF- β , FGF-2, PDGF and VEGF, have been suggested to contribute to fibrotic conditions (Kanbe *et al.* 2000, Hermes *et al.* 2001). Furthermore, mast cell proteases tryptase and chymase have been observed to stimulate fibroblast proliferation and to induce collagen synthesis and collagen fibril formation *in vitro* (Abe *et al.* 1998, Kofford *et al.* 1997). Mast cell histamine may also induce collagen synthesis in fibroblasts (Hatamochi *et al.* 1991). An essential factor for mast cell growth, maturation, survival and activation is the Kit receptor. Significant alterations in Kit receptor expression have been observed in wound healing and in chronic wounds (Huttunen *et al.* 2002).

2.7 Treatment of fibrotic conditions

It has been proposed that fibrosis may not be irreversible tissue damage, and various medical treatments have been attempted to reduce fibrosis.

D-penicillamine has been used to treat systemic scleroderma and extensive localized scleroderma. D-penicillamine chelates copper and thus inhibits the activity of lysyl oxidase, an enzyme involved in collagen fibril and cross-link formation (Oikarinen 1993). Cyclosporine A and extracorporeal photopheresis have also been used to treat scleroderma. These treatments may be capable of reducing collagen synthesis by fibroblasts (Kähäri 1993).

Colchicine has also been used for the treatment of fibrotic conditions. Colchicine is able to reduce collagen synthesis and to increase collagenase production (Oikarinen 1993).

Ultraviolet A has been successfully used for the treatment of localized and systemic scleroderma. UVA has been shown to induce collagenase in fibroblast cultures and in human skin (Scharffetter *et al.* 1991, Gruss *et al.* 1997).

Several studies have demonstrated that glucocorticosteroids inhibit collagen synthesis, and it has been established that glucocorticoids reduce the amount of collagen messenger RNA in human skin fibroblasts (Oikarinen *et al.* 1998). Glucocorticoids also decrease the activity of enzymes needed for collagen biosynthesis (Oikarinen 1993).

The use of the enzyme superoxide dismutase (SOD) as an antifibrotic agent in radiation-induced cutaneous fibrosis has been tested. *In vitro*, SOD has been observed to act as a TGF- β antagonist (Vozenin-Brotans *et al.* 2000). Other proposed antifibrotic agents include the anti-TGF- β antibodies, type I collagen gene expression-inhibiting cytokines such as tumour necrosis factor α (TNF- α) and interferon- γ (IFN- γ), or the use of certain inhibitory intracellular signalling proteins (SMAD-proteins) (Uitto & Kouba 2000).

3 Aims of the present study

Radiotherapy is used to treat breast cancer in order to achieve lower recurrence rates. However, radiotherapy also produces normal tissue damage, which may lead to tissue fibrosis. The mechanisms of radiation-induced fibrosis are not known in detail.

The aims of the present study were as follows:

- To examine the late effect of breast cancer radiotherapy on skin type I and III collagen synthesis, mRNA and metabolism.
- To study the late effect of irradiation on dermal tenascin and elastic fibres, elasticity, blood vessels and physical parameters.
- To examine the late effect of radiotherapy on dermal mast cells and their role in radiation-induced fibrosis.

4 Materials and Methods

4.1 Subjects

This study was carried out at the Department of Dermatology, University of Oulu. A total of 54 subjects participated in the study. The subjects were women who had been treated for breast cancer with operation and radiotherapy at the Oulu University Hospital during the years 1990–1999. The mean age of the subjects was 54 years (range 37–75 years). The time from treatment ranged from 7 to 96 months (mean 25 months). Prior to participating in the study, the subjects underwent control examinations for breast cancer.

Altogether 108 samples of suction blister fluid (SBF), 84 skin punch biopsies and 20 serum samples were collected for the study during the years 1998–1999.

4.2 Suction blister method

The suction blister method was first described in 1964 and the suction blister device developed by Dr. U. Kiistala was introduced in 1968 (Kiistala 1968). This method enables atraumatic separation of viable epidermis from dermis.

In this study, a disposable plastic suction blister device (Dermovac®, Ventipress Oy, Lappeenranta, Finland) was used. This suction blister device consists of an adapter plate with five mutually connected 6mm apertures in a chamber. The device is placed on the skin, and a continuous negative pressure is induced to the chamber. The suction pressure stretches the epidermis upwards, detaching it from the dermis but leaving the basement membrane and thus also the capillaries of the upper dermis intact. As a result, a blister filled with interstitial fluid is formed.

The suction blisters were induced on the radiotherapy-treated skin area of the subjects and on the contralateral non-treated control skin area. A minimum distance of at least 2 cm from the operation scar was maintained. A negative pressure of -50– -60 kPa was used. The time needed for the induction of the blisters was approximately one hour. The

suction blister fluid was collected with a disposable syringe and needle, and the samples were immediately frozen at -20° – -70°C until analysed.

The suction blister method is non-invasive and painless, and local anesthesia is therefore not required. The blisters heal without scarring. A hypopigmentation of the blister sites may persist for some weeks or few months.

4.3 Serum samples

Blood samples were drawn from the cubital vein (I). Two tubes of 10 ml were taken. The serum was separated by centrifugation and the samples were immediately frozen at -20 or -70°C until analysed.

4.4 Physical parameters

4.4.1 Measurement of skin thickness and elasticity

Skin thickness was measured using a 20 MHz ultrasound scanner (Dermascan A; I, II, III or Dermascan C; IV, V, Cortex Technology, Hadsund, Denmark). The transducer of the ultrasound device emits acoustic (ultrasound) pulses to the tissue. To measure the distance between the transducer and the examined tissue, the time lapse between emitting the acoustic pulse and the return of the echo from the tissue is registered. Using an A-mode (A=amplitude; Dermascan A) scanner, the echoes are visualized as a one-dimensional curve. With a B-scan (B=brightness) device, two-dimensional sonographic images can be produced. The B-scan device has been computerized to a so-called C-scan (Dermascan C) (Hoffmann *et al.* 1995). The skin thickness is automatically displayed in millimetres by both devices.

Skin elasticity was measured with a Dermalab® unit (Cortex Technology, Hadsund, Denmark) in accordance to manufacturer's instructions (Gniadecka & Serup 1995, Barel *et al.* 1998). The device used measures the stiffening of the skin by registering the power of vacuum needed to pull up the skin to a certain distance. The values are computed by the device and expressed as MPa.

4.4.2 Measurement of transepidermal water loss and restoration of epidermal barrier function

Transepidermal water loss (TEWL) was measured using Evaporimeter EP1 (Servomed, Stockholm, Sweden). Measurement of TEWL is used for characterization of the skin water barrier function. The evaporimeter quantifies the rate of evaporation of water from

the surface of skin. The probe of the device contains in an open chamber a pair of sensors that measure the relative humidity and temperature at two levels (3 and 9 mm) above the skin. TEWL is expressed as the release of water in grams per square meter per hour (Pinnagoda *et al.* 1990).

Restoration of epidermal barrier function on the wound base was studied in a group of 12 patients using measurements of TEWL, skin blood flow and skin colour and erythema (unpublished). The measurements were performed on suction blister wounds after removal of blister roofs immediately after induction, on day 4 and 1 month after induction.

Skin blood flow was measured from suction blister wounds using a Laser-Doppler flowmeter (Periflux, Perimed, Stockholm, Sweden) (unpublished). Laser-Doppler flowmetry is an optical technique for estimation of microcirculation. A narrow laser light beam (a 632 nm helium-neon source) is directed towards the skin. It penetrates approximately 1 mm into the dermis according to the optical properties of the tissue. The light beam is reflected back from the structures of the tissue. The laser light scattered from moving particles, i.e. the cells in blood vessels, is shifted in frequency according to the Doppler principle. The light reflected back from the tissue is received by the flowmeter detector and converted to an electric signal. The blood perfusion is defined by detecting the concentration and velocity of moving blood cells (erythrocytes), and expressed in arbitrary units (Bircher *et al.* 1994).

To analyse suction blister wound regeneration, measurements of skin colour and erythema were performed on suction blister wounds (unpublished). Minolta Chroma Meter (Osaka, Japan) and DermaSpectrometer (Cortex Technology, Hadsund, Denmark) were used for the measurements. The Minolta Chroma Meter employs a tristimulus (blue, red, green) analysis of light reflected from skin structures. Generally, intense white light covering the entire visible spectrum is emitted from the measuring head of the Minolta Chroma Meter, and the colour of the reflected light is analysed by high-sensitivity photocells (Fullerton *et al.* 1996). The DermaSpectrometer is a spectrophotometric device. It measures the absorbance and reflectance of selected wavelengths of visible light from the skin. Erythema is expressed as an index of haemoglobin relative to melanin. The instrument has a light-emitting source and a photodiode to detect light remitted from the skin (Fullerton *et al.* 1996).

4.5 Analysis of procollagen propeptides of type I and III procollagens in suction blister fluid and serum

4.5.1 Aminoterminal propeptide of type I procollagen (PINP)

The assay for intact amino-terminal propeptide of human type I procollagen (PINP) was described in 1996 (Melkko *et al.* 1996). This assay detects the native, trimeric form of the PINP antigen in serum. Intact PINP assay kit of Orion Diagnostica (Oulunsalo, Finland) was used (I). A 50 µl serum sample was used to determine the PINP level. The reference

values of serum PINP are 19–84 µg/l for women and 20–76 µg/l for men. The intact PINP assay is based on radioimmunoassay technique. Generally, a sample containing an unknown amount of PINP is mixed with standard amounts of a radioactively labelled PINP and PINP antiserum. The labelled standard antigen and unlabelled antigen of the sample then compete for the limited number of binding sites of the antibody. The amount of radioactive PINP in the centrifuged supernatant is reversely proportional to the amount of unlabelled PINP in the sample, and the PINP-concentration of the sample can thus be calculated.

The level of the type I procollagen propeptide (PINP) in SBF was analysed with radioimmunoassay using 5–10 µl of SBF (I–V).

4.5.2 Aminoterminal propeptide of type III procollagen (PIIINP)

The level of PIIINP in serum samples was analysed using the radioimmunoassay of Orion Diagnostica (Oulunsalo, Finland) (I). The assay detects the PIIINP antigens that are the same size or larger than the authentic propeptide (Risteli *et al.* 1988). The reference values for PIIINP for adults are 1.7–4.2 µg/l.

The level of PIIINP was analysed in SBF samples using radioimmunoassay. 5–20 µl of SBF was used for the assay.

4.6 Histology and immunohistochemistry

For histological and immunohistochemical studies, skin samples (punch biopsies of 4 or 6 mm) were obtained under local anaesthesia from the irradiated skin area and the contralateral non-treated skin area of the patients. A minimum distance of at least 2 cm from the operation scar was maintained. Haematoxylin-Eosin (HE) and Verhoeff stainings were performed. For the immunohistochemical studies, anti-PINP, anti-PIIINP, anti-tenascin and anti-human von Willebrand factor (factor VIII, fVIII) antibodies were used.

The samples were fixed in 10% buffered formalin, embedded in paraffin and cut into 4-µm sections. The sections were pretreated with pepsin digestion for anti-PINP and anti-PIIINP stainings. For anti-PINP, a microwave oven heating for 2 min at 95°C in 10 mol/l sodium citrate buffer, pH 6, was also used (Zhu *et al.* 1995). The antibodies for procollagen propeptides PINP and PIIINP were from Dr. Leila Risteli and Dr. Juha Risteli (Department of Clinical Chemistry, Oulu University Hospital, Oulu, Finland). No pretreatment was used for anti-tenascin. Monoclonal anti-tenascin143DB7C8 (Locus, Helsinki, Finland) and polyclonal anti-human von Willebrand factor (Dako A/S, Denmark) were used. The immunohistochemical stainings were performed using an ABC/HRP kit (Dako A/S, Glostrup, Denmark) in accordance with the manufacturer's instructions. For negative controls, the antibody was substituted with phosphate-buffered saline (PBS) solution.

4.7 Computerized digital image analysis

Verhoeff staining was performed to visualize elastic fibres (III). The proportional area of elastic fibres was measured using computerized morphometric analysis (Uitto *et al.* 1983). A Nikon EFD 3 (Japan) light microscope with a 40 x E Plan objective was used. The video signal was analysed with a MCID-M4 image analysis system (Imaging Research Inc., Canada). Six fields were analysed from each section, three from the upper (papillary) and three from the lower (reticular) dermis. The proportional area of elastic fibres in the scan area was calculated. The thickness of epidermis was measured from 10 separate sites of each section and the mean was used for the calculations (III).

4.8 *In situ* hybridization analysis for type I and III collagens

A 372-bp PstI-PvuII fragment of the cDNA for the human pro α 1(I) chain of type I procollagen (Mäkelä *et al.* 1988, Vuorio *et al.* 1987) corresponding to part of the carboxy-terminal propeptide domain of the protein, and a 379-bp PstI-PstI fragment of the cDNA for human pro α 1(III) (Sandberg *et al.* 1989) covering part of the triple-helical as well as the carboxy-terminal telopeptide and propeptide parts of the chain, were inserted in antisense and sense orientations in the polylinker sites of the RNA transcription vector pGEM1. The presence and orientation of the insert were verified by sequencing. The vectors were linearized with suitable enzymes. The transcripts were labelled with digoxigenin-11-UTP (DIG RNA Labeling Kit, Boehringer Mannheim, Germany) for *in situ* hybridization, and a corresponding sense probe was used as a control for non-specific hybridization.

In situ hybridization was performed principally as described previously (Mäkelä *et al.* 1999). Formalin-fixed, paraffin-embedded 6- μ m tissue sections were deparaffinized and pretreated with 0.2 mol/l HCl for 20 minutes at room temperature, followed by a 5-minute wash in DEPC-H₂O and digestion with 20 μ g/ml proteinase K in phosphate-buffered saline (PBS) for 20 minutes at 37°C. To stop the reaction, the sections were dipped in 0.2% glycine in PBS at room temperature and washed twice for 5 minutes. The sections were acetylated in 0.5% acetic anhydride in 0.1M triethanolamide for 2 x 5 minutes at room temperature. The sections were then equilibrated with 4 x standardized saline citrate (SSC) for 15 minutes before prehybridization. The hybridization solution contained 4xSSC, 250 g/ml yeast tRNA, 10mM DTT, 10% dextran sulphate, 2x Denhardt's solution, 50% formamide.

The sections were prehybridized for 2 hours at 55°C. After that, the prehybridization buffer was removed and the hybridization buffer containing 400 ng/ml (Type I collagen) or 300 ng/ml (Type III collagen) digoxigenin (DIG)-labelled antisense or sense probe was denatured at 80°C, applied to each section and hybridized overnight at 55°C.

The following post-hybridization washes were made: 15 minutes in 2 x SSC, 4 x 15 minutes in 0.1 x SSC at 60°C and 5 minutes in 0.1 x SSC at room temperature.

After washing, sections were equilibrated with buffer 1 (containing 100 mM Tris-HCl, 150 mM NaCl, pH 7.5) and blocked for 30 minutes in a solution containing buffer 1, 0.1% Triton-X-100 and 2% normal goat serum. The blocking solution was removed, and

buffer 1 containing alkaline phosphatase (AP) -conjugated anti-digoxigenin (diluted 1:200) (Boehringer Mannheim) was applied to the sections and incubated for 1.5 hours. After washing, AP-anti-digoxigenin was detected with Fast Red solution (Boehringer Mannheim), which yields a red precipitation. The samples were counterstained with Mayer's haematoxylin (Histolab Products AB, Sweden).

After the *in situ* hybridization procedure, the fibroblasts positive for type I and III collagen synthesis were counted under a light microscope (Olympus, BH-2, Japan) using a 40 x objective. The positive cells were counted separately from the upper and lower parts of the dermis. The number of cells was divided by the number of fields counted from each part of the section, and the mean was used for the calculations.

4.9 Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs)

For detecting TIMP-1, TIMP-2, MMP-9 and MMP-2/TIMP-2 complex in SBF (I), serum (I) and skin biopsies (II), an enzyme-linked immunosorbent assay (ELISA) was used (Öberg *et al.* 2000).

ELISAs were performed of 96-well microtiter plates using standard protocols. A polyclonal antibody derived from chicken against each of the analytes was used as a secondary antibody. A peroxidase-labelled anti-chicken-IgG (Chemicon, U.S.A.) was used for detection of the bound secondary antibody. OPD-tablets (KemEnTec, Denmark) were used in visualizing the peroxidase label.

To quantify the level of MMP-2, two ELISAs were used. One measures the free form that is not bound to its inhibitor, and the other detects the MMP-2/TIMP-2 complex. To measure the free MMP-2, the ELISA plate was coated with TIMP-2. After applying the sample and standards, the bound MMP-2 was detected as described above. The MMP-2/TIMP-2 complex was measured by coating the plate with monoclonal anti-TIMP-2 antibody, and detecting the bound complex with a polyclonal anti-MMP-2 antibody. To determine the amount of MMP-9, TIMP-1 and TIMP-2, a monoclonal antibody which recognizes both the free and the complex-bound forms was used. The test plate was coated with the monoclonal antibody, and after adding the samples and standards the bound proteins were detected with polyclonal antibodies as described above (Öberg *et al.* 2000).

4.10 Mast cell analysis

4.10.1 Enzyme-histochemical staining methods for mast cell tryptase and chymase

From the irradiated skin area and the corresponding control skin area, 6-mm punch biopsies were taken under local anaesthesia (V). The biopsies were embedded in the OCT compound and frozen in isopentane. For the staining of mast cell tryptase and chymase, 5- μ m cryosections were fixed in 0.6% formaldehyde and 0.5% acetic acid (pH 7.2) for 10 minutes. Tryptase activity in mast cells was demonstrated enzyme-histochemically with 1 mM Z-Gly-Pro-Arg-MNA as the substrate and 0.5 mg/ml Fast black K salt as the chromogen (pH 7.5), as described previously (Harvima *et al.* 1999). Chymase activity in mast cells was demonstrated enzyme-histochemically with 1 mM Suc-Val-Pro-Phe-MNA and 0.5 mg/ml Fast black K salt (Harvima *et al.* 1999). Mast cells positive for tryptase and chymase activity were counted under a light microscope (Olympus BH-2) using a 0.2 x0.2 ocular grid. The values were expressed per mm².

4.10.2 Immunohistochemical staining for Kit receptor

Cryosections were fixed in cold acetone for 10–15 min. Subsequently, Kit protein was stained with 3 g/ml anti-CD117/Kit mAB (Southern Biotechnology Associates, Inc., Birmingham, AL, U.S.A). The bound antibody was visualized with the avidin-biotin-peroxidase (ABC) technique using the Vectastain Elite ABC kit (Vector) together with 0.05% 3,3'-diaminobenzidizidine tetrahydrochloride, 0.04% nickel chloride and 0.03% hydrogen peroxide (Huttunen *et al.* 2002). The stainings were controlled by using unrelated mouse immunoglobulin.

4.10.3 Immunohistochemical staining method for chymase and chymase inhibitor

A mouse monoclonal anti-chymase antibody (0.1 g/ml) (Biogenesis Ltd., Poole, U.K.) was used to stain mast cell chymase immunohistochemically. In order to stain chymase inhibitor, a rabbit polyclonal anti- α_1 -antichymotrypsin antibody (0.61 g/ml) (Dako, Glostrup, Denmark) was used as previously described (Harvima *et al.* 1999).

4.10.4 Sequential double-staining for TNF- α in mast cells

TNF- α was localized in tryptase-positive mast cells by first staining tryptase enzyme-histochemically and thus identifying mast cells. After photographing 3 sections per slide at 6 sites beneath the epidermis, the stain was dissolved by incubating in 15% Tween 20 solution. After that, the sections were immunostained with 50 g/ml rabbit polyclonal anti-TNF- α antibody (Sera-Lab, Crawley Down, Sussex, UK), and rephotographed at the same sites as previously (Huttunen *et al.* 2000).

4.10.5 Double-staining of mast cells and fibroblasts

The sections were fixed in acetone and sequentially incubated in the blocking solution, mouse monoclonal anti-prolyl-4-hydroxylase antibody solution (5B5 clone, 1:100 dilution, from Dako, Glostrup, Denmark), and biotinylated secondary antibody according to standard histochemical procedure. After that, tryptase was stained enzyme-histochemically with 1 mM Z-Gly-Pro-Arg-MNA as the substrate but using 0.5 mg/ml Fast scarlet R (Sigma) as the chromogen, resulting in orange staining. After staining of the mast cells, the immunohistochemical staining was continued with incubation in the ABC solution according to the manufacturer's instructions (Vector).

4.11 Other assays

Soluble PINP was analysed from 4-mm skin biopsies. Tissue samples were weighed and homogenized into a solution containing 1 ml of phosphate-buffered saline-Tween and kept frozen until analysed. From soluble extracts, PINP was measured by RIA using 50 ml duplicates of extract fluid (II).

The supernatants of skin biopsies were analysed for the concentration of cross-linked carboxyterminal telopeptide of type I collagen (ICTP) and SP4, a peptide structure corresponding to the most aminoterminal part of the carboxyterminal telopeptide of the 1 chain of human type I collagen (II).

Insoluble skin matrices from the skin biopsies were heat-denatured and digested with trypsin. Thereafter the variants of the carboxyterminal telopeptide of type I collagen were separated by high-performance liquid chromatography. The major histidinohydroxy-lysionorleucine (HHL) -cross-linked variant was quantified by the SP4 assay, and the minor pyridinoline analogue (PA) -cross-linked telopeptide was quantified by the ICTP assay (II).

4.12 Ethical considerations

The study protocol had been approved by the ethical committee of the Oulu University Medical Faculty and the Oulu University Hospital. All the subjects gave their informed consent. The study was carried out in accordance with the provisions of the Declaration of Helsinki.

4.13 Statistical analysis

For statistical analyses, the paired samples t-test (I, II, III, IV, V) and Wilcoxon signed ranks test (III) were employed. Statistical Products and Service Solutions (SPSS) for Windows (Microsoft Corp.) was used for the calculations.

5 Results

5.1 Physical parameters

Skin thickness was increased in the irradiated skin area compared with non-treated skin (I, IV). In non-treated skin area, the mean skin thickness values were 1.63 mm (I) and 1.51 mm (IV). In radiotherapy-treated skin area, the mean skin thickness values above the surgical scar were 1.88 mm ($p=0.004$) (I) and 1.78 mm ($p=0.026$) (IV). Below the scar, the values of treated skin were 1.84 mm ($p=0.002$) (I) and 1.60 mm ($p=0.369$) (IV).

The mean value measured by elastometer was slightly increased in irradiated skin, indicating increased skin stiffness. In irradiated skin area the mean values obtained were 15.1 MPa (III) and 16.78 MPa (IV). In control skin area the corresponding values were 13.4 MPa ($p=0.026$) (III) and 14.98 MPa ($p=0.197$) (IV).

No significant difference could be detected in the epidermal regeneration rate between irradiated and control skin as measured by TEWL, skin blood flow and skin colour and erythema on suction blister wounds in a group of 12 patients (unpublished).

5.2 The level of type I and III procollagen propeptides in suction blister fluid

The concentrations of aminoterminal propeptides of type I (PINP) and III (PIIINP) procollagens were markedly increased in suction blister fluid obtained from irradiated skin compared to non-treated skin (I–V). The mean level of PINP in suction blister fluid of irradiated skin was 2.8–3.8 fold higher compared to non-treated skin ($p<0.05$). The mean level of PIIINP in suction blister fluid of irradiated skin was 2.2–3.4 fold higher compared to control skin area ($p<0.05$).

5.3 Immunohistochemical staining and in situ hybridization for type I and III collagens

The amount of fibroblasts positive for PINP was significantly increased in radiotherapy-treated skin, and the increase in fibroblast numbers was prominent in upper parts of the dermis ($p < 0.05$) (I, IV, V). Intracellular PIIINP could not be detected using immunohistochemical staining (I). This is probably due to the fact that the intracellular level of PIIINP is much lower than that of PINP.

The amount of fibroblasts positive for type I collagen mRNA was increased in irradiated skin compared to control skin. The mean number of fibroblasts positive for type I collagen mRNA in the upper dermis of irradiated skin was 14.9, while the corresponding value in non-treated skin was 9.7 ($p = 0.045$). In lower dermis, the number of type I collagen mRNA positive fibroblasts was 9.4 in irradiated skin and 8.4 in control skin ($p = 0.695$) (IV).

The mean number of fibroblasts positive for type III collagen RNA in irradiated skin was 21.0 in upper dermis and 9.9 in lower dermis. In non-treated skin, the corresponding values were 15.6 in upper dermis and 10.0 in lower dermis (IV). The differences were statistically non-significant.

5.4 Total skin collagen, collagen degradation markers ICTP and SP4, and type I collagen cross-link analysis

Soluble PINP was analysed from 4-mm skin biopsies taken from the radiotherapy-treated skin area and the corresponding non-treated skin area of the patients (I). The amount of PINP in irradiated skin was markedly higher compared to non-treated skin ($p = 0.015$).

The concentration of type I collagen degradation marker SP4 was significantly increased in the soluble tissue extracts of skin biopsies of irradiated skin ($p = 0.031$). No statistically significant difference was found between the irradiated and contralateral sides with respect to the soluble ICTP antigen (II).

From 4-mm skin biopsies, the histidinohydroxylsinonorleucine (HHL)- and pyridinoline analogue cross-linked telopeptide of type I collagen were quantified biochemically (II). The amount of cross-linked type I collagen was increased in irradiated skin relative to total collagen, and the type of main cross-link (HHL) was identical to that in normal skin. The average concentration of the HHL-cross-linked variant of type I collagen was 323 ± 119 mmol/mol total amount of collagens in irradiated skin, and 188 ± 117 mmol/mol total amount of collagens in non-treated skin ($p = 0.0001$). The hydroxyproline contents from the biopsies of the irradiated sites were significantly higher.

5.5 MMP and TIMP levels

There was a difference between MMP and TIMP levels obtained from the SBF and those detected from tissue extracts. In suction blister fluid, the mean level of MMP-2/TIMP-2 complex was markedly higher in irradiated skin compared to non-treated skin. The amount of MMP-9 in SBF was below the detection level of the assay used. The mean level of TIMP-1 in SBF was higher in irradiated skin than in non-treated skin ($p=0.017$). The levels of TIMP-2 in SBF were similar in irradiated and control skin ($p=0.382$) (I).

Measured from skin biopsy extracts, however, the concentrations of MMP-2/TIMP-2 complex were significantly lower in irradiated skin compared to non-treated skin ($p=0.008$) (II). There were no significant differences in the levels of TIMP-1 between irradiated skin (0.63 ng/mg) and control skin (0.81 ng/mg). The TIMP-2 levels were 0.78 ng/mg in treated skin and 0.90 ng/mg in control skin. The differences were statistically non-significant (II).

5.6 Serum levels of aminoterminal propeptides of type I and III procollagens (PINP and PIIINP), tissue inhibitors of matrix metalloproteinases (MMPs) 1 and 2 (TIMP-1 and TIMP-2) and MMP-9 and MMP-2/TIMP-2 complexes

The levels of aminoterminal propeptides of type I and III procollagens were within normal range among the subjects. The mean level of PINP in the serum was 35 $\mu\text{g/l}$ in radiotherapy-treated breast cancer patients (reference value 19–84). The mean serum level of PIIINP in the patients was 2.64 $\mu\text{g/l}$ (reference value 1.70–4.20).

Apart from the increased level of MMP-9, the serum levels of the MMPs and TIMPs measured were found normal. The mean serum level of TIMP-1 was 155 $\mu\text{g/l}$ in the patients (reference value 84–186) and the corresponding mean serum level of TIMP-2 was 166 $\mu\text{g/l}$ (reference value 129–222). The level of MMP-2/TIMP-2 complex in serum was 756 $\mu\text{g/l}$ (reference value 475–826) and the level of MMP-9 in serum was 164 $\mu\text{g/l}$ (reference value 9–84).

5.7 Mast cells

The number of tryptase, chymase and Kit-receptor positive mast cells was increased in the upper dermis of irradiated skin (V). The mean number of tryptase positive cells in irradiated skin was 195 in dermal level I, and in non-treated skin the corresponding value was 153 ($p=0.001$). The mean number of chymase positive cells in irradiated skin was 137 in dermal level I, and 85 in dermal level II. In non-treated skin the corresponding values were 91 in dermal level I and 56 in dermal level II ($p=0.009$).

The mean number of Kit receptor positive cells in irradiated skin was 166 in dermal level I, and in control skin the corresponding value was 137 ($p=0.003$). In dermal level II,

the mean number of Kit-receptor positive cells was 58 in irradiated skin and 32 in non-treated skin ($p=0.005$). In dermal level III, the corresponding values were 52 in irradiated skin and 31 in control skin ($p=0.034$). There was no marked difference in the number of cells positive for the chymase inhibitor α_1 -antichymotrypsin between irradiated and non-treated skin. In irradiated skin, 5% of all mast cells were TNF- positive compared to 6% in non-treated skin ($p=0.491$).

In the sections double-stained for mast cell tryptase and prolyl-4-hydroxylase of active fibroblasts, both fibroblasts and mast cells appeared to predominate in the upper dermis of both lesional and non-lesional skin. Tryptase-positive mast cells could occasionally be found in close proximity to fibroblasts (V).

5.8 Elastin, tenascin and factor VIII staining

Elastic fibres were visualized using Verhoeff staining, and their proportional area in the specimen was measured using a computerized image analysis (III). In the upper dermis, the proportional area of elastic fibres was 3.3% in irradiated skin and 3.1% in non-treated skin ($p=0.805$). In the lower dermis, the proportional area of elastic fibres was 5.6% in radiotherapy-treated skin and 5.9% in non-treated skin ($p=0.617$). There was no difference in the morphology or amount of elastic fibres between irradiated and non-treated skin.

Immunohistochemical staining for tenascin was performed using a monoclonal antitenascin (III). In non-treated skin, tenascin expression was found primarily as a narrow band in the papillary dermis below the epidermis, in the blood vessel walls and adjacent to cutaneous appendices. Tenascin expression was not found in the epidermis, lower dermis or subcutaneous fat. In radiotherapy-treated skin, the immunostaining for tenascin beneath the epidermis was more intense, thicker and continuous. Immunohistochemical staining showed an increase in tenascin expression in radiotherapy-treated skin ($p=0.026$).

The number of blood vessels was found to be slightly higher in the upper dermis of irradiated skin compared to non-treated skin. To visualize dermal blood vessels, an immunohistochemical staining for factor VIII (FVIII, von Willebrand factor) was performed (III). The mean number of blood vessels was 19.2 in the upper dermis of irradiated skin and 14.7 in the upper dermis of non-treated skin ($p=0.010$). The corresponding values for lower dermis were 8.2 in irradiated skin and 6.4 in non-treated skin ($p=0.223$).

6 Discussion

6.1 Modulation of skin collagen synthesis by irradiation

Skin collagen synthesis was found to be markedly increased as a consequence of radiotherapy. The level of PINP in suction blister fluid (SBF) reflecting the actual local type I collagen synthesis *in vivo* was increased 2.8–3.8 fold in irradiated skin compared to non-treated skin. The level of PIIINP in SBF, reflecting local type III collagen synthesis in skin, was 2.2–3.4 fold in radiotherapy-treated skin compared to control skin. These findings are in accordance with previous results (Autio *et al.* 1998). The amount of PINP measured from skin biopsies was also significantly increased in irradiated skin, and these values correlated markedly with the level of PINP in SBF samples. The levels of circulating procollagen propeptides remained within normal range, further indicating that radiotherapy caused only local increase of skin type I and III collagen synthesis.

The number of PINP-positive fibroblasts was also significantly increased in irradiated skin, indicating increased type I collagen production in fibroblasts as a result of radiotherapy. Intracellular PIIINP could not be detected immunohistochemically. This could be due to the fact that the intracellular level of PIIINP is much lower than that of PINP.

Type I and III collagen synthesis was also studied on RNA level using ISH. A significantly increased number of fibroblasts positive for type I and III collagen mRNA was found in irradiated skin. There was a significant correlation between the number of type I collagen mRNA positive fibroblasts and the level of PINP in SBF. These findings suggest that type I and III collagen synthesis was increased in radiotherapy-treated skin on both protein and transcriptional level. Also, skin thickness and skin stiffness were found to be increased on the radiotherapy-treated side, which could be due to an accumulation of dermal connective tissue components as a result of irradiation.

It should also be noted that an increase of protein content also results in an increase of protein-bound water in skin. This may also contribute to thickening of skin after radiotherapy (Lahtinen *et al.* 1999).

The concentration of type I collagen degradation marker (SP4) was increased in the soluble biopsy extracts of irradiated skin. There also was a significant correlation between

SP4 and the marker of type I collagen synthesis (PINP), indicating an increased turnover of type I collagen in irradiated skin.

Measured from insoluble tissue digests, an increase of cross-linked type I collagen in irradiated skin could be found. The main cross-link type in irradiated skin was HHL, similarly to that in normal skin (Mechanic *et al.* 1987). This finding suggests that the microstructure of collagen fibres in irradiated skin is conserved. It has also been shown that during normal ageing the content of HHL-cross-linked type I collagen increases in skin (Yamauchi *et al.* 1988). The findings in irradiated skin thus resemble those seen in the ageing process of skin. A similar increase in concentration of HHL-cross-linked type I collagen has also been found in skin samples of scleroderma patients (Ishikawa *et al.* 1998).

The levels of MMP-9, MMP-2/TIMP-2 complex, TIMP-1 and TIMP-2 were determined in both SBF and tissue extracts (Öberg *et al.* 2000). In irradiated skin, the level of MMP-2/TIMP-2 complex was found to be increased in SBF. Measured from tissue extracts, however, the concentration of MMP-2/TIMP-2 complex was lower in irradiated skin compared to non-treated skin. In addition, no significant difference could be found in the levels of TIMP-1 and TIMP-2 measured from tissue extracts, but in SBF the level of TIMP-1 was higher in treated than in non-treated skin. This suggests that no significant alteration of collagen degradation capacity in irradiated skin could be detected using the aforementioned markers.

6.2 Effect of irradiation on skin elastic fibres, tenascin and blood vessels

No difference in the morphological appearance or proportional area of elastic fibres could be found between radiotherapy-treated and non-treated skin. The reason for higher values measured by elastometer in irradiated skin could thus be an accumulation of connective tissue, especially collagens, as a result of irradiation.

The amount of tenascin immunostaining was markedly increased in irradiated skin. The exact role of tenascin *in vivo* is not clearly defined. In normal adult human skin, the expression of tenascin is fairly low (Lightner *et al.* 1989). An increase of tenascin expression has previously been shown in several pathological conditions, such as scleroderma (Lacour *et al.* 1992), and also in wound healing (Latijnhouwers *et al.* 1996) and after topical retinoid treatment (Haapasaari *et al.* 1997). In addition, upregulation of tenascin expression has previously been found in pig skin as a result of irradiation (Geffrotin *et al.* 1998). Accumulation of tenascin could be due to and induction of cytokines by irradiation. TGF- β is a cytokine that has been shown to be induced by irradiation (Barcellos-Hoff 1993), and also to increase tenascin deposition by fibroblasts (Gentilhomme *et al.* 1999).

The number of blood vessels in irradiated skin was slightly increased, and the increase was more pronounced in upper parts of the dermis. The mechanism behind this is not known. These findings could be due to the activation of angiogenetic factors, such as TGF- β , as a consequence of irradiation, or due to the vascular wall damage caused by

irradiation. Despite the increase in their number, all the blood vessels in irradiated dermis may not be functional. In blood flow measurements using Laser-Doppler, no difference between radiotherapy-treated skin and non-treated skin could be found. The mechanism behind this is not known.

6.3 Suction blister wound regeneration

No difference could be detected in restoration of epidermal barrier function rate between radiotherapy-treated and non-treated skin areas, as measured by the reduction of TEWL as well as by measurements of skin blood flow, skin colour and erythema in suction blister bases. This suggests that the epidermal regeneration rate is not affected by the changes in collagen synthesis, and that the epidermal regeneration rate reflecting keratinocyte migration and differentiation remained intact in irradiated skin. This finding is in agreement with previous results on scleredema skin, where collagen synthesis was markedly increased while the restoration of epidermal barrier function was intact (Haapasaari *et al.* 1996).

6.4 Mast cells in irradiated skin

The number of mast cells positive for tryptase, chymase and Kit receptor was significantly increased in the upper dermis of radiotherapy-treated skin compared to non-treated control skin. This finding suggests that mast cells may be one factor contributing to the increased collagen synthesis and fibrosis induced by radiotherapy. This is in line with previous studies. Previously, mast cells have been found to be involved in the pathogenesis of scleroderma and in wound healing, and several mast cell cytokines such as TGF- β , have been associated with fibrogenesis (Irani *et al.* 1992, Trabucchi *et al.* 1988, Kanbe *et al.* 2000, Hermes *et al.* 2001). The most abundant proteins in mast cells, tryptase and chymase, have been shown to induce collagen synthesis and to stimulate fibroblast proliferation (Abe *et al.* 1998). Histamine, a mast cell mediator, is also able to induce collagen synthesis in skin fibroblasts (Hatamochi *et al.* 1991).

7 Conclusions

1. In the present study, skin type I and III collagen synthesis was found to be significantly increased as a consequence of breast cancer radiotherapy. Type I collagen turnover and cross-linking was also increased as a result of irradiation. The collagen degradation capacity and its inhibitors measured by the levels of MMP-9, MMP-2/TIMP-2 complex, TIMP-1 and TIMP-2 were not significantly altered in irradiated skin. The findings indicate that the balance of collagen metabolism was not severely disturbed in the group of patients studied. However, the results provide new information on the mechanisms of radiation-induced fibrosis.
2. No difference in skin elastic fibres could be detected histologically between radiotherapy-treated and non-treated skin. The higher skin stiffness values observed in irradiated skin could be due to an accumulation of connective tissue in irradiated skin. The amount of blood vessels in factor VIII-stained sections was slightly higher in irradiated skin. This could be due to an activation of angiogenic factors as a result of irradiation or tissue hypoxia. The mechanism behind this is not known.
3. The amount of tenascin immunostaining was markedly increased in irradiated skin. The increased tenascin expression could be due to an activation of cytokines as a consequence of irradiation.
4. The changes in collagen synthesis rate did not alter the restoration of epidermal barrier function rate in irradiated skin. This indicates that keratinocyte migration and differentiation remained intact in irradiated skin.
5. The number of mast cells positive for tryptase, chymase and Kit receptor was increased in the upper dermis of radiotherapy-treated skin. The number of mast cells with chymase activity was also higher in irradiated skin. This suggests that mast cells have a role in radiation-induced fibrosis.

References

- Abe M, Kurosawa M, Ishikawa O, Miyachi & Kido H (1998) Mast cell tryptase stimulates both human dermal fibroblast proliferation and type I collagen production. *Clin Exp Allergy* 28:1509–1517.
- Aimes RT & Quigley JP (1995) Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific $\frac{3}{4}$ - and $\frac{1}{4}$ - length fragments. *J Biol Chem* 270:5872–5876.
- Annala A-P, Risteli L, Koivukangas V, Autio P, Risteli J & Oikarinen A (1993) Measurement of collagen metabolism in skin diseases. A review of old and new techniques and their clinical applications. *Eur J Dermatol* 3:696–703.
- Araya J, Maruyama M, Sassa K, Fujita T, Hayashi R, Matsui S, Kashii T, Yamashita N, Sugiyama E & Kobayashi M (2001) Ionizing radiation enhances matrix metalloproteinase-2 production in human lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 280:30–38.
- Archambeau JO, Pezner R & Wasserman T (1995) Pathophysiology of irradiated skin and breast. *Int J Radiat Oncol Biol Phys* 31:1171–1185.
- Autio P, Saarto T, Tenhunen M, Elomaa I, Risteli J & Lahtinen T (1998) Demonstration of increased collagen synthesis in irradiated human skin in vivo. *Br J Cancer* 77:2331–2335.
- Barcellos-Hoff MH (1993) Radiation-induced transforming growth factor β and subsequent extracellular matrix reorganization in murine mammary gland. *Cancer Res* 53:3880–3886.
- Barel AO, Lambrecht R & Clarys P (1998) Mechanical function of the skin: State of the art. In: Elsner P, Barel AO, Berardesca E, Gabard B & Serup J (eds) *Skin bioengineering*. Karger AG Basel p 69–83.
- Bateman JF, Mascara T, Chan D & Cole WG (1984) Abnormal type I collagen metabolism by cultured fibroblasts in lethal perinatal osteogenesis imperfecta. *Biochem J* 217:103–115.
- Bentzen SM & Overgaard M (1994) Clinical radiobiology and normal-tissue morbidity after breast cancer treatment. *Adv Radiat Biol* 18:25–51.
- Bircher A, de Boer EM, Agner T, Wahlberg JE & Serup J (1994) Guidelines for measurement of cutaneous blood flow by laser Doppler flowmetry. A report from the Standardization Group of the European Society of Contact Dermatitis. *Contact Dermatitis* 30:65–72.
- Border WA & Noble NA (1994) Mechanisms of disease: transforming growth factor (beta) in tissue fibrosis. *NEJM* 331:1286–1292.
- Border WA, Ruoslahti E (1992) Transforming growth factor- β in disease: The dark side of tissue repair. *J Clin Invest* 90:1–7.
- Bristow J, Tee MK, Gitelman SE, Mellon SH & Miller WL (1993) Tenascin-X: a novel extracellular matrix protein encoded by the human XB gene overlapping P450c21B. *J Cell Biol* 122:265–278.

- Brown-Augsburger P, Tisdale C, Broekelmann T, Sloan C & Mecham RP (1995) Identification of an elastin cross-linking domain that joins three peptide chains. Possible role in nucleated assembly. *J Biol Chem* 270:17778–17783.
- Burchardt ER, Heke M, Kauschke SG, Harjes P, Kohlmeyer J, Kroll W, Schauer M, Schroeder W & Voelker M (1998) Epitope-specific monoclonal antibodies against human c-terminal procollagen $\alpha 1$ (III)-propeptide. *Matrix Biol* 17:673–677.
- Carnemolla B, Leprini A, Borsi L, Querz G, Urbini S & Zardi L (1996) Human Tenascin-R. *J Biol Chem* 271:8157–8160.
- Canney PA & Dean S (1990) Transforming growth factor beta: a promotor of late connective tissue injury following radiotherapy? *Br J Radiol* 63:620–623.
- Chapman HA, Riese RJ & Shi G-P (1997) Emerging roles for cysteine proteases in human biology. *Annu Rev Physiol* 59:63–88.
- Chiquet M & Fambrough DM (1984) Chick myotendinous antigen I. A monoclonal antibody as a marker for tendon and muscle morphogenesis. *J Cell Biol* 98:1926–1936.
- Chiquet-Ehrismann R (1995) Tenascins, a growing family of extracellular matrix proteins. *Experientia* 51:853–862.
- Cirino G, Napoli C, Bucci M & Cicala C (2000) Inflammation-coagulation network: are serine protease receptors the knot? *Trends Pharmacol Sci* 21:170–172.
- Dalkowski A, Schuppan D, Orfanos CE & Zouboulis CC (1999) Increased expression of tenascin C by keloids in vivo and in vitro. *Br J Dermatol* 141:50–56.
- Debelle L & Tamburro AM (1999) Elastin: molecular description and function. *Int J Biochem Cell Biol* 31:261–272.
- Elder D, Elenitsas R, Johnson B Jr & Jaworsky C (1997) Radiation dermatitis. In: Elder D, Elenitsas R, Jaworsky C & Johnson B Jr (eds) *Lever's histopathology of the skin*. Lippincott-Raven Publishers Philadelphia-New York p 313–316.
- Erickson HP (1993) Tenascin-C, tenascin-R and tenascin-X: a family of talented proteins in search of functions. *Curr Opin Cell Biol* 5:869–876.
- Eyre D (1984) Cross-linking in collagen and elastin. *Ann Rev Biochem* 53:717–748.
- Everts V, van der Zee E, Creemers L & Beertsen W (1996) Phagocytosis and intracellular digestion of collagen, its role in turnover and remodelling. *Histochem J* 28:229–245.
- Fazio MJ, Mattei MG, Passage E, Chu ML, Black D, Solomon E, Davidson JM & Uitto J (1991) Human elastin gene: new evidence for localization to the long arm of chromosome 7. *Am J Hum Genet* 48:696–703.
- Fazio MJ, Olsen DR, Kuivaniemi H, Chu ML, Davidson JM, Rosenbloom J & Uitto J (1988) Isolation and characterization of human elastin cDNAs, and age-associated variation in elastin gene expression in cultured skin fibroblasts. *Lab Invest* 58:270–277.
- Fehlauer F, Tribius S, Höller U, Rades D, Kuhlmeier A, Bajrovic A & Alberti W (2003) Long-term radiation sequelae after breast-conserving therapy in women with early-stage breast cancer: an observational study using the LENT-SOMA scoring system. *Int J Radiat Oncol Biol Phys* 55:651–658.
- Fisher GJ, Choi HC, Bata-Csorgo Z, Shao Y, Datta S, Wang ZQ, Kang S & Voorhees JJ (2001) Ultraviolet irradiation increases matrix metalloproteinase-8 protein in human skin in vivo. *J Invest Dermatol* 117:219–226.
- Fitzgerald J & Bateman JF (2001) A new FACIT of the collagen family: COL21A1. *FEBS* 505:275–280.
- Fleischmajer R, Perlish JS, Burgeson RE, Shaikh-Bahai F & Timpl R (1990) Type I and type III collagen interactions during fibrillogenesis. *Ann N Y Acad Sci* 580:161–175.
- Fullerton A, Fischer T, Lahti A, Wilhelm K-P, Takiwaki H & Serup J (1996) Guidelines for measurement of skin colour and erythema. *Contact Dermatitis* 35:1–10.

- Geffroin C, Tricaud Y, Crechet F, Castelli M, Lefaix JL & Vaiman M (1998) Unlike Tenascin-X, Tenascin-C is highly up-regulated in pig cutaneous and underlying muscle tissue developing fibrosis after necrosis induced by very high-dose gamma radiation. *Radiat Res* 149:472–481.
- Ghosh AK (2002) Factors involved in the regulation of type I collagen gene expression: implication in fibrosis. *Exp Biol Med* 227:301–314.
- Gentilhomme E, Neveux Y, Lebeau J, Desmouliere A, Bergier J, Schmitt D & Haftek M (1999) Modulation of a fibrotic process induced by transforming growth factor beta-1 in dermal equivalents. *Cell Biol Toxicol* 15:229–238.
- Gniadecka M & Serup J (1995) Suction chamber method for measurement of skin mechanical properties: the Dermaflex®. In: Serup J & Jemec GBE (eds) *Handbook of non-invasive methods and the skin*. CRC Press, Inc. Boca Raton p 329–334.
- Gruss C, Reed JA, Altmeyer P, McNutt NS & Kerscher M (1997) Induction of interstitial collagenase (MMP-1) by UVA-1 phototherapy in morphea fibroblasts. *Lancet* 350:1295–1296.
- Gulcher JR, Nies DE, Alexakos MJ, Ravikant NA, Sturgill ME, Marton LS & Stefansson K (1991) Structure of the human hexabrachion (tenascin) gene. *Proc Natl Acad Sci* 88:9438–9442.
- Haapasaari K-M, Kallioinen M, Tasanen K, Risteli J, Palatsi R & Oikarinen A (1996) Increased collagen propeptides in the skin of a scleredema patient but no change in re-epithelialisation rate. *Acta Derm Venereol* 76:305–309.
- Haapasaari K-M, Kallioinen M, Tasanen K, Sutinen M, Annala A-P, Risteli J & Oikarinen A (1997) Effect of topical tretinoin on non-sun-exposed human skin connective tissue: induction of tenascin but no major effect on collagen metabolism. *Br J Dermatol* 136:891–900.
- Hagios C, Brown-Luedi M & Chiquet-Ehrismann R (1999) Tenascin-Y, a component of distinctive connective tissues, supports muscle cell growth. *Exp Cell Res* 253:607–617.
- Harvima IT, Haapanen L, Ackermann L, Naukkarinen A, Harvima RJ & Horsmanheimo M (1999) Decreased chymase activity is associated with increased levels of protease inhibitors in mast cells of psoriatic lesions. *Acta Derm Venereol* 79:98–104.
- Hatamochi A, Ueki H, Mauch C & Krieg T (1991) Effect of histamine on collagen and collagen mRNA production in human skin fibroblasts. *J Dermatol Sci* 2:407–412.
- Haukipuro K, Melkko J, Risteli L, Kairaluoma M & Risteli J (1991) Synthesis of type I collagen in healing wounds in humans. *Ann Surg* 213:75–80.
- Hermes B, Welker P, Feldmann-Böddcker I, Krüger-Krasagakis S, Hartmann K, Zuberbier T & Henz BM (2001) Expression of mast cell growth modulating and chemotactic factors and their receptors in human cutaneous scars. *J Invest Dermatol* 116:387–393.
- Hoegl L, Korting HC & Klebe G (1999) Inhibitors of aspartic proteases in human diseases: molecular modeling comes of age. *Pharmazie* 54:319–329.
- Hoffmann K, Röchling A, Stecker M, Dirting K, el Gammal S, Hoffmann A & Altmeyer P (1995) High-Frequency sonography of skin diseases. In: Serup J & Jemec GBE (eds) *Handbook of noninvasive methods and the skin*. CRC Press, Inc. Boca Raton p 269–278.
- Holli K, Blanco G, Dean P, Hietanen P, Isola J, Joensuu H, Kataja V, Kumpulainen E, Lammi U-K, Saaristo R, Valavaara R, von Smitten K & Österman H (2002) Rintasyövän hoito. *Duodecim* p 1–19.
- Huttunen M, Aalto M-L, Harvima RJ, Horsmanheimo M & Harvima IT (2000) Alterations in mast cells showing tryptase and chymase activity in epithelializing and chronic wounds. *Exp Dermatol* 9:258–265.
- Huttunen M, Naukkarinen A, Horsmanheimo M & Harvima IT (2002) Transient production of stem cell factor in dermal cells but increasing expression of Kit receptor in mast cells during normal wound healing. *Arch Dermatol Res* 294:324–330.
- Irani AM, Gruber BL, Kaufman LD, Kahaleh MB & Schwartz LB (1992) Mast cell changes in scleroderma. Presence of MC_T cells in the skin and evidence of mast cell activation. *Arthritis Rheum* 35:933–939.

- Ishikawa O, Kondo A & Miyachi Y (1998) Mature type of skin collagen crosslink, histidinohydroxylysinonorleucine, is significantly increased in the skin of systemic sclerosis patients. *Arthritis rheum* 41:376–377.
- Joestner A & Faissner A (2001) The structure and function of tenascins in the nervous system. *Matrix biol* 20:13–22.
- Jones FS & Jones PL (2000) The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling. *Develop Dyn* 218:235–259.
- Johansen J, Bentzen SM, Overgaard J & Overgaard M (1996) Relationship between the in vitro radiosensitivity of skin fibroblasts and the expression of subcutaneous fibrosis, telangiectasia, and skin erythema after radiotherapy. *Radiother Oncol* 40:101–109.
- Johansson S, Svensson H & Denekamp J (2000) Timescale of evolution of late radiation injury after postoperative radiotherapy of breast cancer patients. *Int J Radiat Oncol Biol Phys* 48:745–750.
- Kadler K, Holmes D, Trotter J & Chapman J (1996) Collagen fibril formation. *Biochem J* 316:1–11.
- Kähäri V-M (1993) Activation of dermal connective tissue in scleroderma. *Ann Med* 25:511–518.
- Kähäri V-M & Saarialho-Kere U (1997) Matrix metalloproteinases in skin. *Exp Dermatol* 6:199–213.
- Kähäri V-M & Saarialho-Kere U (1999) Matrix metalloproteinases and their inhibitors in tumour growth and invasion. *Ann Med* 31:34–45.
- Kanbe N, Kurosawa M, Nagata H, Yamashita T, Kurimoto F & Miyachi Y (2000) Production of fibrogenic cytokines by cord blood-derived cultured human mast cells. *J Allergy Clin Immunol* 106:S85–S90.
- Kielty CM, Sherratt MJ & Shuttleworth CA (2002) Elastic fibres. *J Cell Sci* 115:2817–2828.
- Kiistala U (1968) Suction blister device for separation of viable epidermis from dermis. *J Invest Dermatol* 50:129–137.
- Kivirikko K, Laitinen O & Prockop D (1967) Modifications of a specific assay for hydroxyproline in urine. *Analytical Biochem* 19:249–255.
- Koch M, Foley JE, Hahn R, Zhou P, Burgeson RE, Gerecke DR & Gordon MK (2001) I(XX) collagen, a new member of the collagen subfamily, fibril-associated collagens with interrupted triple helices. *J Biol Chem* 276:23120–23126.
- Kofford MW, Schwartz LB, Schechter NM, Yager DR, Diegelmann RF & Graham MF (1997) Cleavage of type I procollagen by human mast cell chymase initiates collagen fibril formation and generates a unique carboxyl-terminal propeptide. *J Biol Chem* 272:7127–7131.
- Koukoulis GK, Gould VE, Bhattacharyya A, Gould JE, Howedy AA & Virtanen I (1991) Tenascin in normal, reactive, hyperplastic, and neoplastic tissues: biologic and pathologic implications. *Hum Pathol* 22:636–643.
- Kuutti-Savolainen ER & Kero M (1979) Enzymes of collagen biosynthesis in skin and serum in dermatological diseases. *Enzymes of the skin. Clin Chim Acta* 15:43–51.
- Lacour JP, Vitetta A, Chiquet-Ehrismann R, Pisani A & Ortonne JP (1992) Increased expression of tenascin in the dermis in scleroderma. *Br J Derm* 127:328–334.
- Lahtinen T, Nuutinen J, Alanen E, Turunen M, Nuortio L, Usenius T & Hopewell J (1999) Quantitative assessment of protein content in irradiated human skin. *Int J Radiat Oncol Biol Phys* 43:635–638.
- Latijnhouwers M, Bergers M, Ponc M, Dijkman H, Andriessen M & Schalkwijk J (1997) Human epidermal keratinocytes are a source of tenascin-C during wound healing. *J Invest Dermatol* 108:776–783.
- Levi-Schaffer F & Pe'er J (2001) Mast cells and angiogenesis. *Clin Exp Allergy* 31:521–524.
- Lightner VA, Gumkowski F, Bigner DD & Erickson HP (1989) Tenascin/hexabrachion in human skin: biochemical identification and localization by light and electron microscopy. *J Cell Biol* 108:2483–2493.
- Lopez-De Leon A & Rojkind M (1985) A simple micromethod for collagen and total protein determination in formalin-fixed paraffin-embedded sections. *J Histochem Cytochem* 33:737–743.

- Mackie EJ, Halfter W & Liverani D (1988) Induction of tenascin in healing wounds. *J Cell Biol* 107:2757–2767.
- Mackie EJ (1994) Tenascin in connective tissue development and pathogenesis. *Perspect Dev Neurobiol* 2:125–132.
- Mackie EJ & Tucker RP (1999) The tenascin-C knockout revisited. *J Cell Sci* 112:3847–3853.
- Mäkelä JK, Raassina A, Virta A & Vuorio E (1988) Human pro α 1(I) collagen: cDNA sequence for the C-propeptide domain. *Nucleic Acid Res* 16:349.
- Mäkelä M, Larjava H, Pirilä E, Maisi P, Salo T, Sorsa T & Uitto VJ (1999) Matrix metalloproteinase 2 (gelatinase A) is related to migration of keratinocytes. *Exp Cell Res* 251:67–78.
- Malkinson FD & Hanson WR (1991) Radiobiology of the skin. In: Goldsmith LA (ed) *Physiology, biochemistry, and molecular biology of the skin*. Oxford University Press, Inc. New York p 976–1016.
- Martin M, Lefaix J-L, Pinton P, Crechet F & Daburon F (1993) Temporal modulation of TGF- β 1 and β -actin gene expression in pig skin and muscular fibrosis after ionizing radiation. *Radiat Res* 134:63–70.
- Martin M, Remy J & Daburon F (1989) Abnormal proliferation and aging of cultured fibroblasts from pigs with subcutaneous fibrosis induced by gamma irradiation. *J Invest Dermatol* 93:497–500.
- Mayr NA, Riggs CE, Saag KG, Wen BC, Pennington EC & Hussey DH (1997) Mixed connective tissue disease and radiation toxicity. A case report. *Cancer* 79:612–618.
- Mecham RP & Heuser JE (1991) The elastic fiber. In: Hay, ED (ed) *Cell biology of extracellular matrix*. Plenum press, New York p 79–109.
- Mechanic GL, Katz EP, Henmi M, Noyes C & Yamauchi M (1987) Locus of a histidine-based, stable trifunctional, helix to helix collagen cross-link: stereospecific collagen structure of type I skin fibrils. *Biochemistry* 26:3500–3509.
- Melkko J, Hellevik T, Risteli L, Risteli J & Smedsrød B (1994) Clearance of NH₂-terminal propeptides of types I and III procollagen is a physiological function of the scavenger receptor in liver endothelial cells. *J Exp Med* 179:405–412.
- Melkko J, Kauppila S, Niemi S, Risteli L, Haukipuro K, Jukkola A & Risteli J (1996) Immunoassay for intact amino-terminal propeptide of human type I procollagen. *Clin Chem* 42:947–954.
- Meric F, Buchholz TA, Mirza NQ, Vlastos G, Ames FC, Ross MI, Pollock RE, Singletary SE, Feig BW, Kuerer HM, Newman LA, Perkins GH, Strom EA, McNeese MD, Hortobagyi GN & Hunt KK (2002) Long-term complications associated with breast-conservation surgery and radiotherapy. *Ann Surg Oncol* 9:543–549.
- Mori Y, Kähäri V-M & Varga J (2002) Scleroderma-like cutaneous syndromes. *Current Rheumatol Reports* 4:113–122.
- Moy RL, Moy LS, Matsuoka LY, Bennett RG & Uitto J (1988) Selectively enhanced procollagen gene expression in sclerosing (morphea-like) basal cell carcinoma as reflected by elevated pro 1(I) and pro 1(III) procollagen messenger RNA steady-state levels. *J Invest Dermatol* 90:634–638.
- Nagase H & Woessner Jr JF (1999) Matrix metalloproteinases. *J Biol Chem* 274:21491–21494.
- Nuutinen J, Väänänen A, Lahtinen T, Turunen M, Remes S & Alanen E (2000) Radiobiological depth of subcutaneous induration. *Radiother Oncol* 55:187–190.
- Öberg Å, Höyhty M, Tavelin B, Stenling R & Lindmark G (2000) Limited value of preoperative serum analyses of matrix metalloproteinases (MMP-2, MMP-9) and tissue inhibitors of matrix metalloproteinases (TIMP-1, TIMP-2) in colorectal cancer. *Anticancer Res* 20:1085–1091.
- Oikarinen A (1993) Drugs acting on connective tissue. In: Lapière CM & Krieg T (eds) *Connective tissue diseases of the skin*. Marcel Dekker, Inc. New York, Basel, Hong Kong, p 361–375.
- Oikarinen A (1997) Basic aspects of collagen metabolism. In: Altmeyer P, Hoffmann K & Stücker M (eds) *Skin cancer and UV radiation*. Springer-Verlag Berlin, Heidelberg p 77–93.

- Oikarinen A, Autio P, Kiistala U, Risteli L & Risteli J (1992) A new method to measure type I and III collagen synthesis in human skin in vivo: demonstration of decreased collagen synthesis after topical glucocorticoid treatment. *J Invest Dermatol* 98:220–225.
- Oikarinen A, Haapasaari K-M, Sutinen M & Tasanen K (1998) The molecular basis of glucocorticoid-induced skin atrophy: topical glucocorticoid apparently decreases both collagen synthesis and the corresponding collagen mRNA level in human skin in vivo. *Br J Dermatol* 139:1106–1110.
- Oikarinen A, Palatsi R, Adomian GE, Oikarinen H, Clark JG & Uitto J (1984) Anetoderma: biochemical and ultrastructural demonstration of an elastin defect in the skin of three patients. *J Am Acad Dermatol* 11:64–72.
- Okada Y, Nara K, Kawamura K, Matsumoto T, Nakanishi I, Fujimoto N, Sato H & Seiki M (1995) Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: implications for bone resorption. *Lab Invest* 72:311–322.
- Olsen BR (1991) Collagen biosynthesis. In: Hay ED (ed) *Cell biology of extracellular matrix*. Plenum press, New York p 177–220.
- Paglia L, Wilczek J, de Leon LD, Martin GR, Hörlein D & Müller P (1979) Inhibition of procollagen cell-free synthesis by amino-terminal extension peptides. *Biochemistry* 18:5030–5034.
- Peacock J, Ashton A, Bliss J, Bush C, Eady J, Jackson C, Owen R, Regan J & Yarnold J (2000) Cellular radiosensitivity and complication risk after curative radiotherapy. *Radiother Oncol* 55:173–178.
- Peltonen S, Hentula M, Hägg P, Ylä-Outinen H, Tuukkanen J, Lakkakorpi J, Rehn M, Pihlajaniemi T & Peltonen J (1999) A novel component of epidermal cell-matrix and cell-cell contacts: transmembrane protein type XIII collagen. *J Invest Dermatol* 113:635–642.
- Perlish JS, Timpl R & Fleischmajer R (1985) Collagen synthesis regulation by the aminopropeptide of procollagen I in normal and scleroderma fibroblasts. *Arthritis Rheum* 28:647–651.
- Pezner RD, Lorant JA, Terz J, Ben-Ezra J, Odom-Maryon T & Luk KH (1992) Wound-healing complications following biopsy of the irradiated breast. *Arch Surg* 127:321–324.
- Pierce SM, Recht A, Lingos T, Abner A, Vicini F, Silver B, Herzog A & Harris JR (1992) Long-term radiation complications following conservative surgery (cs) and radiation therapy (rt) in patients with early stage breast cancer. *Int J Radiat Oncol Biol Phys* 23:915–932.
- Pinnagoda J, Tupker RA, Agner T & Serup J (1990) Guidelines for transepidermal water loss (TEWL) measurement. *Contact Dermatitis* 22:164–178.
- Porock D, Nikoletti S & Kristjanson L (1999) Management of radiation skin reactions: literature review and clinical application. *Plastic Surgical Nursing* 19:185–193.
- Porock D, Kristjanson L, Nikoletti S, Cameron F & Pedler P (1998) Predicting the severity of radiation skin reactions in women with breast cancer. *Oncol Nurs Forum* 25:1019–1029.
- Prockop DJ & Kivirikko KI (1995) Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem* 64:403–434.
- Risteli J, Niemi S, Kauppila S, Melkko J & Risteli L (1995) Collagen propeptides as indicators of collagen assembly. *Acta Orthop Scand suppl* 66:183–188.
- Risteli J, Niemi S, Trivedi P, Mäentausta O, Mowat AP & Risteli L (1988) Rapid equilibrium radioimmunoassay for the amino-terminal propeptide of human type III procollagen. *Clin Chem* 34:715–718.
- Risteli J & Risteli L (1995) Analysing connective tissue metabolites in human serum. Biochemical, physiological and methodological aspects. *J Hepatol* 22:77–81.
- Risteli L (1993) How collagens are broken down. In: *Assay of collagen metabolism*. Orion Diagnostica Helsinki, Finland p 16–17.
- Risteli L (1993) Quantifying type III collagen synthesis – assay of the aminoterminale propeptide of type III procollagen (PIIINP). In: *Assay of collagen metabolism*. Orion Diagnostica Helsinki Finland p. 43–44.

- Robertson JM, Clarke DH, Pevzner MM & Matter RC (1991) Breast conservation therapy. Severe breast fibrosis after radiation therapy in patients with collagen vascular disease. *Cancer* 68:502–508.
- Rodemann HP & Bamberg M (1995) Cellular basis of radiation-induced fibrosis. *Radiother Oncol* 35:83–90.
- Rohde H, Bruckner P & Timpl R (1983) Immunochemical properties of the aminopropeptide of procollagen type III. *Eur J Biochem* 135:197–202.
- Rosenbloom J, Abrams WR & Mecham R (1993) Extracellular matrix 4: the elastic fiber. *FASEB J* 7:1208–1218.
- Rossert JA & Garret LA (1995) Regulation of type I collagen synthesis. *Kidney Int* 47:Suppl 49:S34–S38.
- Sandberg M, Mäkelä JK, Multimäki P, Vuorio T & Vuorio E (1989) Construction of a human pro α 1(III) collagen cDNA clone and localization of type III collagen expression in human fetal tissues. *Matrix* 9:82–91.
- Schaffer JV, Carroll C, Dvoretzky I, Huether MJ & Girardi M (2000) Postirradiation morphea of the breast. Presentation of two cases and review of the literature. *Dermatol* 200:67–71.
- Schalkwijk J, Van Vlijmen I, Oosterling B, Perret C, Koopman R, Van Den Born J & Mackie EJ (1991) Tenascin expression in hyperproliferative skin diseases. *Br J Dermatol* 124:13–20.
- Schalkwijk J, Zweers M, Steijlen P, Dean W, Taylor G, van Vlijmen I, van Haren B, Miller W & Bristow J (2001) A recessive form of the Ehlers-Danlos syndrome caused by tenascin-X deficiency. *N Engl J Med* 345:1167–1175.
- Scharffetter K, Wlaschek M, Hogg A, Bolsen K, Schothorst A, Goerz G, Krieg T & Plewig G (1991) UVA irradiation induces collagenase in human dermal fibroblasts *in vitro* and *in vivo*. *Arch Dermatol Res* 283:506–511.
- Smedsrød B, Melkko J, Risteli L & Risteli J (1990) Circulating c-terminal propeptide of type I procollagen is cleared mainly via the mannose receptor in liver endothelial cells. *Biochem J* 271:345–350.
- Soini Y, Pöllänen R, Autio-Harminen H & Lehto VP (1997) Tenascin expression in lichen sclerosus. *Int J Gynecol Pathol* 16:313–318.
- Stenlicht MD & Werb Z (2001) How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 17:463–516.
- Takehara K (2000) Growth regulation of skin fibroblasts. *J Dermatol Sci* 24:S70–S77.
- Tasanen K, Hämäläinen E-R, Palatsi R & Oikarinen A (1996) Quantification of pro α 1(I) collagen mRNA in skin biopsy specimens: levels of transcription in normal skin and in granuloma annulare. *J Invest Dermatol* 107:314–317.
- Thornton SC, Walsh BJ, Bennett S, Robbins JM, Foulcher E, Morgan GW, Penny R & Breit SN (1996) Both *in vitro* and *in vivo* irradiation are associated with induction of macrophage-derived fibroblast growth factors. *Clin Exp Immunol* 103:67–73.
- Tokarek R, Bernstein EF, Sullivan F, Uitto J & Mitchell JB (1994) Effect of therapeutic radiation on wound healing. *Clinics in Dermatology* 12:57–70.
- Trabucchi E, Radaelli E, Marazzi M, Foschi D, Musazzi M, Veronesi AM & Montorsi W (1988) The role of mast cells in wound healing. *Int J Tissue React* 10:367–372.
- Travis EL (2001) Organizational response of normal tissues to irradiation. *Semin Radiat Oncol* 11:184–196.
- Trojanowska M, LeRoy EC, Eckes B & Krieg T (1998) Pathogenesis of fibrosis: type I collagen and the skin. *J Mol Med* 76:266–274.
- Turesson I, Nyman J, Holmberg E & Odén A (1996) Prognostic factors for acute and late skin reactions in radiotherapy patients. *Int J Radiat Oncol Biol Phys* 36:1065–1075.
- Uitto J (1970) A method for studying collagen biosynthesis in human skin biopsies *in vitro*. *Biochim Biophys Acta* 201:438–445.

- Uitto J & Bernstein E (1998) Molecular mechanisms of cutaneous aging: connective tissue alterations in the dermis. *J Invest Dermatol* 3:41–44.
- Uitto J & Kouba D (2000) Cytokine modulation of extracellular matrix gene expression: relevance to fibrotic skin diseases. *J Dermatol Sci* 25:S560–S569.
- Uitto J, Paul JL, Brockley K, Pearce RH & Clark JG (1983) Elastic fibers in human skin: quantitation of elastic fibers by computerized digital image analyses and determination of elastin by radioimmunoassay of desmosine. *Lab Invest* 49:499–505.
- Vermeer BJ, Reman FC & van Gent CM (1979) The determination of lipids and proteins in suction blister fluid. *J Invest Dermatol* 73:303–305.
- Vozenin-Brotons MC, Sivan V, Gault N, Renard C, Geffrotin C, Delanian S, Lefaix JL & Martin M (2001) Antifibrotic action of Cu/Zn SOD is mediated by TGF- β 1 repression and phenotypic reversion of myofibroblasts. *Free Radic Biol Med* 30:30–42.
- Vrhovski B & Weiss AS (1998) Biochemistry of tropoelastin. *Eur J Biochem* 258:1–18.
- Vu TH & Werb Z (2000) Matrix metalloproteinases: effectors of development and normal physiology. *Genes & Dev* 14:2123–2133.
- Vuorio T, Mäkelä JK, Kähäri VM & Vuorio E (1987) Coordinated regulation of type I and type III collagen production and mRNA levels of pro α 1(I) and pro α 2(I) collagen in cultured morphea fibroblasts. *Arch Dermatol Res* 279:154–160.
- Weber P, Montag D, Schachner M & Bernhardt RR (1998) Zebrafish tenascin-W, a new member of the tenascin family. *J Neurobiol* 35:1–16.
- Wiestner M, Krieg T, Hörlein D, Glanville RW, Fietzek P & Müller PK (1979) Inhibiting effect of procollagen peptides on collagen biosynthesis in fibroblast cultures. *J Biol Chem* 254:7016–7023.
- Wu CH, Donovan CB & Wu GY (1986) Evidence for pretranslational regulation of collagen synthesis by procollagen propeptides. *J Biol Chem* 1986 261:10482–10484.
- Yamauchi M, Woodley DT & Mechanic GL (1988) Aging and cross-linking of skin collagen. *Biochem Biophys Res Comm* 152:898–903.
- Zhou L-J, Ono I & Kaneko F (1997) Role of transforming growth factor- β 1 in fibroblasts derived from normal and hypertrophic scarred skin. *Arch Dermatol Res* 289:646–652.
- Zhu GG, Risteli L, Mäkinen M, Risteli J, Kauppila A & Stenbäck F (1995) Immunohistochemical study of type I collagen and type I pN-collagen in benign and malignant ovarian neoplasms. *Cancer* 75:1010–1017.

Original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I Riekkö R, Jukkola A, Sassi M-L, Höyhtyä M, Kallioinen M, Risteli J & Oikarinen A (2000) Modulation of skin collagen metabolism by irradiation: collagen synthesis is increased in irradiated human skin. *Br J Dermatol* 142: 874–880.
- II Sassi M-L, Jukkola A, Riekkö R, Höyhtyä M, Risteli L, Oikarinen A & Risteli J (2001) Type I collagen turnover and cross-linking are increased in irradiated skin of breast cancer patients. *Radiother Oncol* 58:317–323.
- III Riekkö R, Jukkola A, Oikarinen A & Kallioinen M (2001) Radiation therapy induces tenascin expression and angiogenesis in human skin. *Acta Derm Venereol* 81:329–333.
- IV Riekkö R, Parikka M, Jukkola A, Salo T, Risteli J & Oikarinen A (2002) Increased expression of collagen type I and III in human skin as a consequence of radiotherapy. *Arch Dermatol Res* 294:178–184.
- V Riekkö R, Harvima I, Jukkola A, Oikarinen A (2004) The production of collagen and the activity of mast cell chymase increase in human skin after irradiation therapy. *Experimental Derm* 13:364–371.

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