SYNTHESIS AND DEGRADATION OF MUSCLE COLLAGEN DURING IMMOBILIZATION, GLUCOCORTICOID TREATMENT AND IN NEUROMUSCULAR DISEASES

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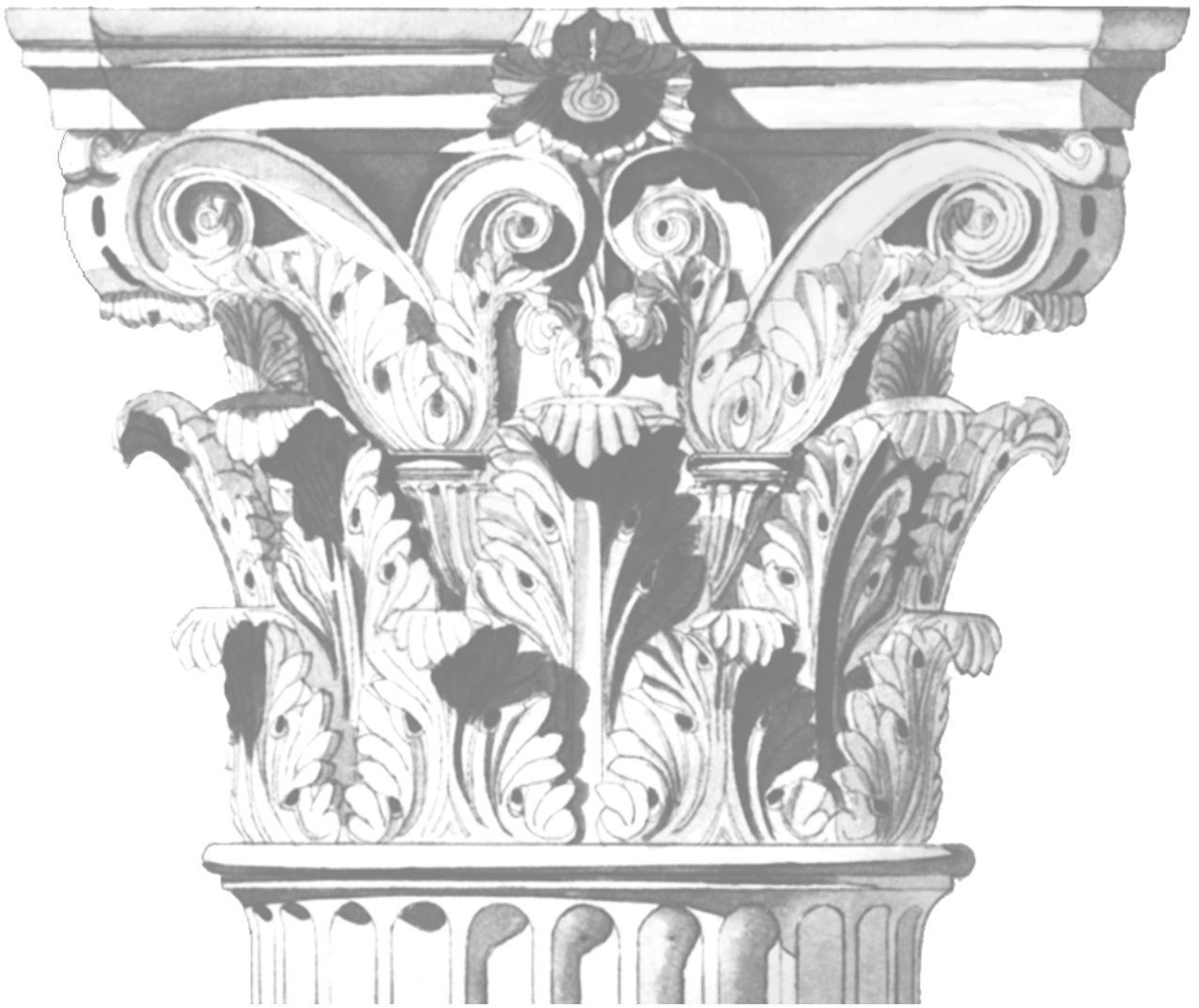

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Abstract

To investigate the turnover of type IV collagen in skeletal muscle in conditions where muscle function is impaired, type IV collagen and proteins regulating its degradation were studied during 1, 3 and 7 days of immobilization, 3- and 10-day glucocorticoid treatment and in neuromuscular diseases. In addition, fibrillar type I and III collagens were studied during immobilization and in neuromuscular diseases. The mRNA levels of type I, III and IV collagens were decreased during immobilization and during 10-day dexamethasone treatment. Gene expression and quantity of (pro)MMP-2 was increased during immobilization but decreased during dexamethasone treatment. The expression of TIMP-2 was decreased both during immobilization and dexamethasone treatment. Decreased gene expression and increased degradation caused decreased concentration of type IV collagen, suggesting net degradation of type IV collagen during immobilization. While the gene expression and degradation were decreased during dexamethasone treatment, the amount of type IV collagen was not changed. Dexamethasone thus seemed to slow down the turnover of type IV collagen. Decreased mRNA levels of collagens and prolyl 4-hydroxylase suggest decreased biosynthesis of collagens during immobilization. The mRNA levels of collagens I, III and IV were increased in polyneuropathy and polymyositis. The concentration and staining intensity of type IV collagen was increased in polyneuropathy, as was also the quantity and staining intensity of (pro)MMP-9. The results suggest accumulation of type IV collagen in the basement membranes of muscle cells and capillaries in polyneuropathy muscles. Lengthened position during immobilization partly prevented the atrophy and changes in collagen metabolism in plantarflexors. Endurance running was effective in preventing muscle atrophy during dexamethasone treatment, but exercise did however fail to prevent the changes observed in type IV collagen synthesis and degradation.

Keywords: collagen, exercise, glucocorticoids, immobilization, matrix metalloproteinases, neuromuscular diseases, skeletal muscle, tissue inhibitor of metalloproteinases

To Anssi, Roope and Veeti

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Oulu, November 2003

Anne Ahtikoski

Abbreviations

cDNA	complementary deoxyribonucleic acid
ECM	extracellular matrix
EDL	extensor digitorum longus muscle
GM	gastrocnemius muscle
Hyp	hydroxyproline
IIINTP	aminoterminal telopeptide of type III collagen
kDa	kilodalton
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
PINP	aminoterminal propeptide of type I collagen
PIIINP	aminoterminal propeptide of type III collagen
proMMP	latent proenzyme form of MMP
P4-H	prolyl 4-hydroxylase
RIA	radioimmunoassay
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SD	standard deviation
SOL	soleus muscle
TA	tibialis anterior muscle
TIMP	tissue inhibitor of metalloproteinases
7-S	N-terminal domain of type IV collagen molecule

List of original articles

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

I Ahtikoski AM, Koskinen SOA, Virtanen P, Kovanen V, Takala TES. Regulation of synthesis of fibrillar collagens in rat skeletal muscle during immobilization in shortened and lengthened position. Acta Physiol Scand 172: 131-140, 2001.

II Ahtikoski AM, Koskinen SOA, Virtanen P, Kovanen V, Risteli J, Takala TES. Synthesis and degradation of type IV collagen in rat skeletal muscle during immobilization in shortened and lengthened positions. Acta Physiol Scand 177: 473-481, 2003.

III Ahtikoski AM, Riso E-M, Koskinen SOA, Risteli J, Takala TES. Regulation of type IV collagen synthesis and degradation in fast and slow muscles during dexamethasone treatment and exercise. Pflügers Archiv – Eur J Physiol. In press.

IV Ahtikoski AM, Tuominen H, Korpelainen JT, Takala TES, Oikarinen A. Synthesis and degradation of collagens in neuromuscular diseases. Submitted.

Contents

1 Introduction

Interstitial collagen types I and III, and basement membrane type IV collagen are the most abundant collagens in skeletal muscle. Intramuscular collagens connect muscle fibers together and to the tendons at the ends of muscles. They support the muscle tissue and distribute forces of muscular contractions. Their concentration is regulated by gene expression, posttranslational modifications and degradation. (Takala & Virtanen 2000) The proteolytical degradation of collagens occurs mainly through the activity of matrix metalloproteinases (MMPs). MMP expression is regulated by their synthesis, and inhibition by specific tissue inhibitors of metalloproteinases, TIMPs. (Visse & Nagase 2003)

Since the adaptability of skeletal muscle to changes in loading is very high, alterations of physical activity cause remodeling of both intracellular contractile components as well as the extracellular matrix (ECM). Disuse causes muscle atrophy by active intra- and extracellular processes. Immobilization is one of the most common experimental models of disuse, although denervation, suspension and hypogravity are also widely used. (Takala & Virtanen 2000) Catabolic hormones, such as glucocorticoids, and chronic inflammation are also known to cause muscle atrophy (Hickson & Marone 1993). Although these various conditions all result in loss of muscle function, their effects on muscle ECM appear to be differently regulated.

The purpose of the present study was to investigate the changes in collagen synthesis and degradation during immobilization, glucocorticoid treatment and neuromuscular diseases. Lengthened position during immobilization, and exercise during glucocorticoid treatment were studied as possible compensatory conditions. While immobilization is known to cause decreased biosynthesis of collagens (Takala & Virtanen 2000), the role of MMPs and TIMPs in the degradation of type IV collagen as well as type IV collagen content during immobilization has not been studied previously. Glucocorticoids are widely used in clinical practice, and they are known to cause changes in the contractile components of skeletal muscle (Hickson & Marone 1993). However, no studies are available of their effects on collagen synthesis and degradation. Although MMPs and TIMPs have been studied recently in neuromuscular diseases, the focus has so far been on inflammatory myopathies: polymyositis, dermatomyositis and inclusion body myositis

(Dalakas 2001). The expression of MMPs or the concentration of type IV collagen has not previously been studied in polyneuropathy.

2 Review of the literature

2.1 Extracellular matrix in skeletal muscle

The extracellular matrix (ECM) is formed by complex molecular networks, which determine the architecture of a tissue and regulate various biological processes (Aumailley & Gayraud 1998), e.g. differentiation of skeletal muscle cells (Melo *et al*. 1996). Skeletal muscle ECM is organized in three levels: the epimysium surrounds the entire skeletal muscle, the perimysium surrounds muscle bundles consisting of a variable number of muscle cells, and the endomysium outlines the individual muscle fibers (Borg & Caulfield 1980) (Fig. 1). The most abundant structural components of ECM are collagens. At present, 21 different collagen types have been identified (Aumailley & Gayraud 1998, Koch *et al*. 2001, Fitzgerald & Bateman 2001).

2.2 Fibril forming and associated collagen types in skeletal muscle

The epimysium consists mainly of type I collagen with minor amounts of type III collagen (Light & Champion 1984, Järvinen *et al.* 2002). Equal amounts of both collagen types are found in the perimysium. In the endomysium, type III collagen is the predominant form and only trace amounts of type I collagen are found (Light & Champion 1984, Järvinen *et al.* 2002). Slow muscles contain more type I collagen than type III collagen, the proportion of type III collagen being greater in fast muscles (Miller *et al.* 2001). Collagens I and III are fibril forming and serve as a supportive structure in muscle tissue. They attach myocytes to each other and muscle bundles to each other (Järvinen *et al*. 2002). Also nerves and capillaries are surrounded and attached to muscle by collagen (Järvinen *et al.* 2002). Type V collagen is also fibril forming and can be found in the endo- and perimysium in smaller amounts than the collagen types I and III (Light & Champion 1984). Collagens III and V are known to copolymerize with type I collagen and they may have a role in collagen fiber diameter regulation (Birk & Mayne 1997, Fleischmajer *et al.* 1990a,b, Birk *et al.* 1990). Type V collagen is postulated to

form the core of the fibrils, and collagens I and III copolymerize around this core (Fig. 2) (Aumailley & Gayraud 1998). Type II and XI collagens, which are also fibril forming, are detected in skeletal muscle only at mRNA level (Sandberg *et al.* 1993). Because type V and XI collagens form heterotypic molecules it is proposed that instead of being separate collagen types, they can be considered as a single kind of collagen (Kleman *et al.* 1992, Mayne *et al.* 1993, Prockop & Kivirikko 1995). Fibril associated collagens with interrupted triple helixes (FACIT) types XII and XIV are located only in the perimysium (Listrat *et al.* 2000), although their distribution is closely related to type I collagen and they are also found in the epimysium and endomysium during embryonic development (Wälchli *et al.* 1994). These FACIT collagens associate with the surface of interstitial collagen fibrils and possibly act as molecular bridges among fibrils or between fibrils and other components of the ECM (Fig. 2) (Wälchli *et al.* 1994). Although mRNAs of the other members of FACIT subfamily (IX, XVI, XIX, XXI) are detected in skeletal muscle, the respective proteins have not been found (Lai & Chu 1996, Bönnemann *et al.* 2000, Sumiyoshi *et al.* 2001, Chou & Li 2002).

2.3 Collagens in the basement membrane of skeletal muscle

Basement membrane (BM) is a highly specialized sheet of connective tissue surrounding individual muscle fibers, blood vessels, Schwann's cells and spindle capsule cells. Components of the BM are regulators of many biological activities such as cell growth, differentiation and migration which influence tissue development and repair (Erickson & Couchman 2000). Integrins attach muscle cells to ECM and serve as force-transmitters between ECM and contracting components inside the muscle cells. They connect laminin to the cell membrane to form the inner layer of basement membrane (Fig. 2) (Aumailley & Gayraud 1998). Type IV collagen molecules form a mesh-like structure outside the laminin layer and give stability to the BM (Kühn 1995). Laminin and type IV collagen are connected to each other by nidogen-1 in muscular basement membranes (Ries *et al.* 2001, Salmivirta *et al.* 2002). N-terminal 7-S domains of four type IV collagen molecules are covalently joined together (Risteli *et al.* 1980), while C-terminal noncollagenous (NC1) domains covalently connect two collagen IV molecules (Than *et al.* 2002).

Type VI collagen interacts with type IV (Kuo *et al.* 1997) and type I collagens (Bonaldo *et al.* 1990), providing a link between basement membranes and the surrounding matrix (Fig. 2). Collagens XV and XVIII comprise the multiplexin subfamily of nonfibrillar collagens (Erickson & Couchman 2000). They are located in the basement membrane zone and are known to bind nidogen-1, laminin-1-nidogen-1 complex, perlecan and fibulin (Myers *et al.* 1996, Sasaki *et al.* 2000, Tomono *et al.* 2002). Collagens XV and XVIII may have a role in stabilizing muscle cells (Eklund *et al.* 2001), although they also have an important physiological role as precursors of endostatins, which inhibit angiogenesis (O'Reilly *et al.* 1997, Sasaki *et al.* 2000).

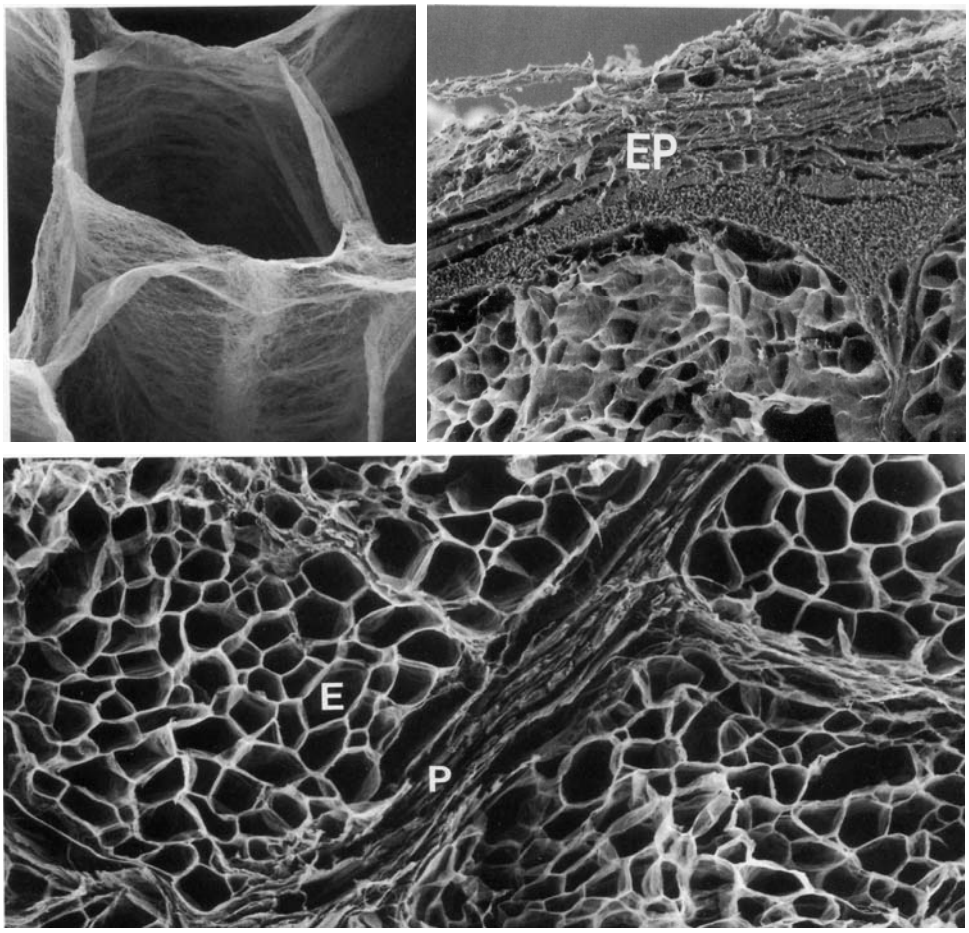

Fig. 1. Scanning electron micrographs of epimysium (EP, top right), perimysium (P, bottom) and endomysium (E, top left and bottom) of bovine semitendinosus muscle. Reprinted from Nishimura et al. (1994). Used with permission from S. Karger AG.

Type XIII collagen is a transmembrane protein (Hägg *et al.* 1998) capable of binding certain basement membrane proteins (Fig. 2) (Tu *et al.* 2002). Therefore it probably has a role as a link between muscle cell and its basement membrane (Kvist *et al.* 2001). Type XIII collagen is concentrated in the myotendinous junctions (Hägg *et al.* 2001), supporting the proposal of its important role in giving mechanical strength to cell-matrix junctions (Tu *et al.* 2002).

2.4 Collagen synthesis in skeletal muscle

A characteristic property of collagens is the formation of triple helixes composed of three polypeptide chains. Fibril forming collagens consist of uninterrupted triple helixes, but other collagens have one or more triple helical domain of various length. In skeletal muscle, collagens are expressed principally by fibroblasts, and their biosynthesis is characterized by the presence of an extensive number of co- and posttranslational modifications of the polypeptide chains (Fig. 3). (Prockop & Kivirikko 1995) Gross fractional synthesis rate for collagen is about 5 %/day in skeletal muscles of young adult rats (Mays *et al.* 1991), whereas the fractional synthesis rate for total protein is about 11-15 %/day (Goldspink *et al.* 1986).

Fibril forming collagens share numerous genetic features. One common feature of the genes is the major triple helical domain of each chain coded by 42 exons, each beginning with a complete codon for glycine. Repeated -Gly-X-Y- tripeptide units enable triple helix formation. Type I collagen consists of two α1(I) chains and one α2(I) chain, while type III collagen is a homotrimer of three α1(III) chains. The structure of type IV collagen genes is distinctly different from those of fibril forming collagens. The collagenous domain of type IV collagen is longer than in the fibril forming collagens; it is however frequently interrupted with noncollagenous sequences. Most type IV collagen molecules consist of a combination of α1(IV) and α2(IV) chains, although combinations of α3(IV) and α4(IV), as well as α5(IV) and α6(IV) chains are found in some basement membranes. (Prockop & Kivirikko 1995)

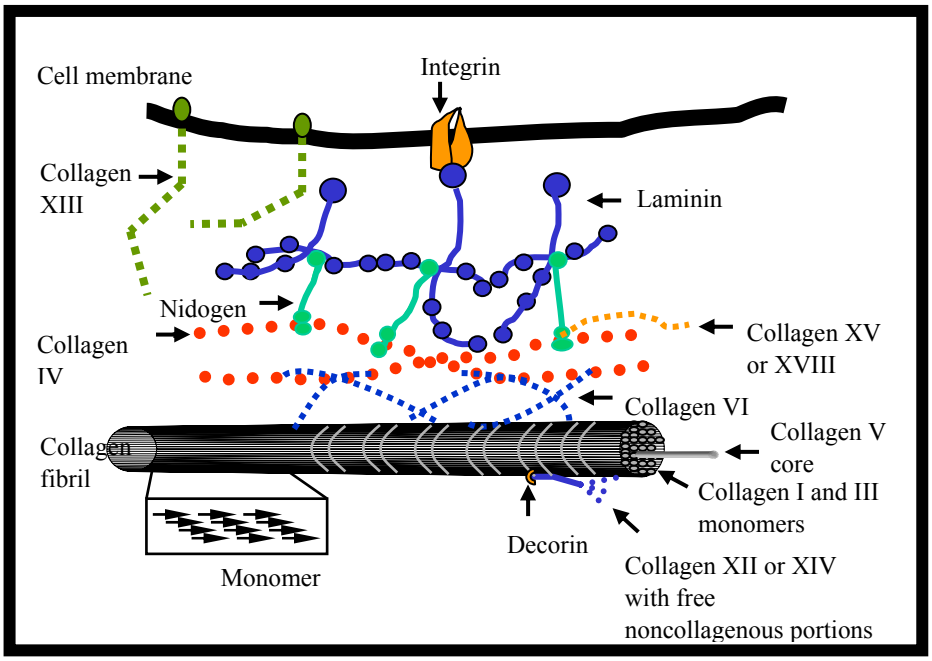

Fig. 2. Collagens in skeletal muscle. (Kuo *et al.*1997, Aumailley & Gayraud 1998 and Tu *et al.* 2002)

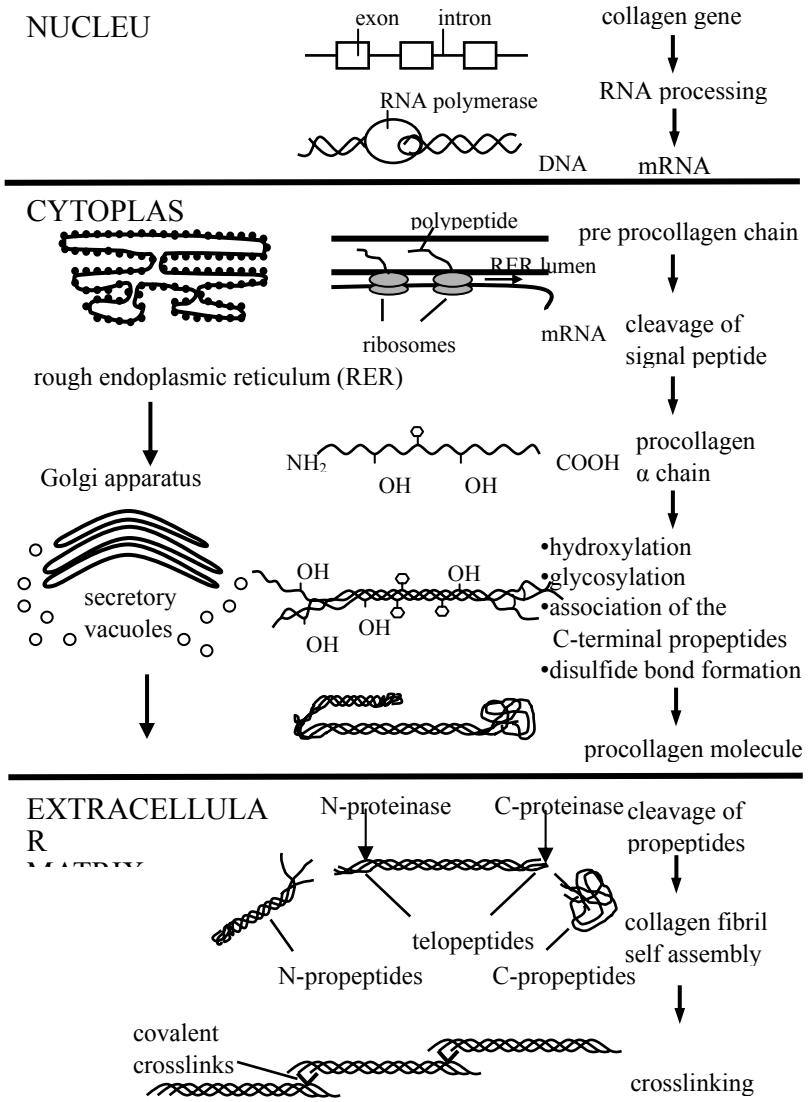

Fig. 3. Synthesis of fibrillar collagens.

2.4.1 Intracellular events

After transcription, mRNA is extensively processed and then translated in rough endoplasmic reticulum (Fig. 3). The first step in intracellular processing of the polypeptide chain is the cleavage of signal peptides. Then the proline and lysine residues

in Y-position are hydroxylated to 4-hydroxyproline and hydroxylysine by prolyl 4-hydroxylase and lysyl hydroxylase. A few X-position proline residues are hydroxylated to 3-hydroxyproline. Galactose and/or glucose are added to some of the hydroxylysine by hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase (GGT). Fibrillar collagens have C- and N-terminal propeptides, in which mannose-rich oligosaccharides are added while processing. Formation of the collagen trimer begins by association of the C-terminal propeptides. Triple helix is formed from a C-terminal nucleus towards the N-terminus in a zipper-like manner. Formation of intra- and interchain disulfide bonds stabilizes the structure. The processing and assembly of fibrillar and nonfibrillar collagens is principally the same, although many nonfibrillar collagens contain N- and /or C-terminal domains that are not removed and therefore not called propeptides. Different collagens may have special features in their synthesis, e.g. chain association and folding of type I and IV collagens may involve a collagen-specific stress protein, heat shock protein 47 (Hsp47). (Laurent 1987, Prockop & Kivirikko 1995, Kadler *et al.* 1996)

2.4.2 Extracellular events

Fibrillar collagens are secreted as soluble procollagens, which have a trimeric globular C-propeptide domain and a trimeric helical N-propeptide domain. These procollagens are converted to collagen by cleavage of terminal propeptides by procollagen N- and C-proteinases (Fig. 3). Collagen then spontaneously self-assembles into fibrils. Stabilization of the fibrils is provided by covalent cross-links generated by conversion of some of the lysine and hydroxylysine residues to aldehyde derivates by lysyl oxidase. (Laurent 1987, Prockop & Kivirikko 1995, Kadler *et al.* 1996)

Type IV collagen molecules form their network with different processes. The N-terminal 7-S domains of four type IV collagens are covalently joined together, while the C-terminal globular domains (NC1) of two separate type IV collagen molecules are joined together by disulfide bonds. Tight meshwork is formed by irregularly branching lateral associations of the triple helical regions. (Yurchenco & O´Rear 1994)

2.5 Degradation of collagens

Collagens can be degraded prior to or after their secretion from the cell. Secreted collagen is degraded mainly by two different routes: proteolytic and phagocytotic. Proteolytic degradation occurs mainly through matrix metalloproteinase (MMP) activity. Magrophages remove ECM components, however also fibroblasts are able to the phagocytosis and degradation of collagen fibrils (Everts *et al.* 1996). Initial fragmentation of insoluble collagens occurs through mechanical wear, the action of free radicals and proteinase cleavage. Degradation is continued by specific proteinases and the resulted collagen fragments are phagocytozed by cells and processed by lysosomal enzymes.

(Cimpean & Caloianu 1997) The proportion of newly synthesized collagen degraded is about 26 % per day in young adult rats (Mays *et al.* 1991), and the most recently synthesized collagen seems to be more susceptible to degradation than mature collagen (Laurent 1987).

2.5.1 MMPs

Matrix metalloproteinases (MMPs) are important components in many biological and pathological processes because of their ability to degrade ECM components. They probably have a key role in tumor invasion, metastasis and angiogenesis. In addition to their role in ECM remodeling, MMPs may regulate paracrine signals by inactivating directly e.g. angiotensins I and II, bradykinin and substance P. MMPs can also degrade fibrinogen and inactivate Factor XII, suppressing the mechanisms for blood clotting. Because of their role in degradation of interstitial elastic fibers, MMPs appear to have important roles in the formation of pulmonary emphysema and intracranial and aortic aneurysms. (Sternlicht & Werb 2001) Up till now, 24 different vertebrate MMPs have been identified, of which 23 have been found in humans (Table 1) (Visse & Nagase 2003). Sequence homology with MMP-1, cysteine switch motif PRCGXPD in the propeptide and the zinc-binding motif HEXGHXXGXXH in the catalytic domain are the special features that assign proteinases to this family (Visse & Nagase 2003). Even though many MMPs are known to have wide and overlapping substrate specificity, MMPs are usually categorized according to their main substrates into collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and others.

MMP-1, MMP-8, MMP-13 and MMP-18 are collagenases with the ability to cleave the native helical structure of interstitial collagens I, II and III (Cimpean & Caloianu 1997, Kähäri & Saarialho-Kere 1999, Li *et al.* 2000, Visse & Nagase 2003). Cleavage products are then susceptible to the action of other MMPs (Cimpean & Caloianu 1997).
Gelatinases (MMP-2 and MMP-9) degrade denatured collagen, gelatin, native type IV, V and VII collagens as well as other ECM components (Collier *et al.* 1988, Wilhelm *et al.* 1989, Trocmé *et al.* 1998, Visse & Nagase 2003). MMP-2 also digests fibrillar type I and II collagens (Aims & Quigley 1995, Patterson *et al.* 2001).

Although stromelysin-1 (MMP-3) and -2 (MMP-10) share substrate specificities for ECM components and they both activate proMMP-1, MMP-3 is proteolytically more efficient than MMP-10 (Visse & Nagase 2003). The third stromelysin, MMP-11, differs from the other stromelysins by its sequence and substrate specificity and it is therefore sometimes grouped with “other MMPs” (Visse & Nagase 2003).

Matrilysins (MMP-7 and MMP-26) are the smallest MMPs. In addition to the ECM components digested by them, MMP-7 can also process cell surface molecules (Visse & Nagase 2003).

Table 1. Family of matrix metalloproteinases (MMPs) with their major substrates and modulators.

MMP	Major substrates	Activators	Inducers
MMP-1/ Interstitial collagenase	Collagens I, II, III, X gelatin	Plasmin MMP-3, -7, -10	TNFα, IL-1β PDGF, EGF
MMP-2/ Gelatinase A	Gelatin, laminin, fibronectin, elastin collagens I, II, IV, V, VII	MT-MMP MMP-1, -13	TGFβ
MMP-3/ Stromelysin	Proteoglycans, fibronectin, gelatin, proMMP-1, collagens III, IV, V, IX	Plasmin	TNFα, IL-1β EGF
MMP-7/ Matrilysin	Gelatin, fibronectin, collagen IV proteoglycan, proMMP-1	Plasmin	LPS
MMP-8/ Collagenase-2	Collagens I, II, III, gelatin	MMP-3, -7, -10	TNFα, IL-1β
MMP-9/ Gelatinase B	Gelatin, proteoglycans, elastin fibronectin, collagens IV, V, VII	Plasmin MMP-3,-13,-26[1]	TGFβ, TNFα IL-1β, LPS, EGF
MMP-10/ Stromelysin-2	Gelatin, fibronectin, proMMP-1 collagens III, IV, V, IX	Plasmin	TGFα, EGF TNFα
MMP-11/ Stromelysin-3	Gelatin	Furin	TGFβ, EGF, IL-6, PDGF
MMP-12/ Metallo-elastase	Elastin, collagen IV, laminin		TNFα, IL-1β PDGF
MMP-13/ Collagenase-3	Collagens I, II, III	Plasmin, MT-MMP MMP-2, -3, -10	LIF, TNFα, IL-1β
MMP-14/ MT1-MMP	Collagens I, II, III ProMMP-2, proMMP-13		TNFα, IL-1β EGF
MMP-15/ MT2-MMP	ProMMP-2, fibronectin, gelatin		Stretch
MMP-16/ MT3-MMP	ProMMP-2, collagen III, gelatin		
MMP-17/ MT4-MMP	Gelatin, fibrin, fibrinogen		
MMP-18/ Collagenase-4	Collagens I, II, III		
MMP-19	Gelatin, collagen IV, laminin[2]		phorbol ester
MMP-20/ Enamelysin	Amelogenin, gelatin	MT1-MMP	
MMP-21	To be determined		
MMP-22	Gelatin		
MMP-23/ Cysteine array MMP	To be determined		

Table 1. Continued

MMP	Major substrates	Activators	Inducers
MMP-24/ MT5-MMP	ProMMP-2, gelatin, fibronectin		
MMP-25/ MT6-MMP	ProMMP-2, collagen IV, gelatin		IL-8
MMP-26/ Matrilysin-2	Gelatin, collagen IV, fibronectin[1]		
MMP-28/ Epilysin	To be determined	furin	

TGFβ = transforming growth factor β; TNFα = tumor necrosis factor α; IL-1β = interleukin-1β; EGF = epithelial growth factor; LPS = lipopolysaccharide; PDGF = platelet derived growth factor; LIF = leukemia inhibitory factor. [1]Uría & López-Otín 2000, [2]Stracke et al. 2000. Other references mentioned in the text.

Six membrane-type MMPs (MT-MMPs) have been characterized. With the exception of MT4-MMP, they all have a broad spectrum of substrate specificity, and they are all capable of activating proMMP-2 (Hernandez-Barrantes *et al.* 2002, Visse & Nagase 2003). The expression of MT5-MMP (MMP-24) is restricted to brain, while the other MT-MMPs are more widely expressed (Visse & Nagase 2003). For their pericellular fibrinolytic activity, MT-MMPs have an important role in angiogenesis (Sternlicht & Werb 2001).

Seven MMPs are currently classified into the group of "other MMPs". MMP-12 is mainly expressed in macrophages and it degrades a large variety of proteins (Shapiro *et al.* 1993). MMP-19 is a novel member identified from patients with rheumatoid arthritis, therefore initially named RASI-1 (Kolb *et al.* 1997). MMP-20 is currently considered a tooth-specific MMP, with the expression strictly restricted to dental cells *in vivo* (Palosaari *et al.* 2003). MMP-22 is known to be expressed in chicken embryo fibroblasts, and it seems to digest gelatine (Yang & Kurkinen 1998). MMP-23 is a transmembrane protein expressed in reproductive tissue (Visse & Nagase 2003). The expression patterns of the newest member of the MMP family, MMP-28, suggest functions in skin hemostasis and wound repair (Illman *et al.* 2003).

The expression of MMPs is primarily regulated at the level of transcription, while the proteolytic activity is regulated by latent precursor activation and inhibition of activity. Cytokines, growth factors and corticosteroids are known to induce or repress the transcription of MMP genes (See Table 1 for details) (Windsor *et al.* 1993, Birkedal-Hansen 1995, Cimpean & Caloianu 1997, Morin *et al.* 1999, Singer *et al.* 1999, Feinberg *et al.* 2000, Li *et al.* 2000, Delany & Canalis 2001, Kang *et al.* 2001, Mauch *et al.* 2002). On the other hand, MMPs are known to regulate the activity of many cytokines and growth factors (Sternlicht & Werb 2002, Visse & Nagase 2003). While plasmin is a potent activator of various MMPs, the zymogens can also be activated by other enzymes as well as other MMPs (Bassi *et al.* 2000, Kotra *et al.* 2001, Palosaari *et al.* 2002, Illman *et al.* 2003). In addition to its role in inhibition of MMPs, TIMP-2 also has a role in the activation of MMP-2 (Strongin *et al.* 1995). The modulators of the latest MMPs are presently unknown.

MMP-1, MMP-2, MMP-9 and MMP-16 are expressed in bovine skeletal muscle on mRNA level (Balcerzak *et al.* 2001), and MMP-1, MMP-2 and MMP-9 proteins in human skeletal muscle (Singh *et al.* 2000). Although MMP-13 and MMP-15 expression are not observed in the bovine muscle, their mRNAs are found in the RNA extracted from skeletal muscle connective tissue cells (Balcerzak *et al.* 2001). Therefore it seems that MMP-13 and MMP-15 are expressed in skeletal muscle in tiny amounts. In muscle diseases, also MMP-3, MMP-7, MMP-10 and MMP-11 proteins have been reported in human skeletal muscle (Schoser & Blottner 1999, Kieseier *et al.* 2001). In skeletal muscle, MMPs are primarily expressed by fibroblasts, although some level of expression has been found to occur also in satellite cells (Guérin & Holland 1995, Balcerzak *et al.* 2001). MMP-9 is expressed in skeletal muscle mainly by infiltrating leukocytes (Kherif *et al.* 1999, Kieseier *et al.* 2001).

2.5.1.1 MMP inhibition by TIMPs

MMP activity can be specifically inhibited by tissue inhibitors of metalloproteinases (TIMPs) as well as by non-specific inhibitors including e.g. α2-macroglobulin, RECK and tissue factor pathway inhibitor-2 (Baker *et al.* 2002, Visse & Nagase 2003). Four TIMPs have been characterized and observed to regulate MMP activity during tissue remodeling (Docherty *et al.* 1985, Stetler-Stevenson *et al.* 1989, Apte *et al.* 1995, Greene *et al.* 1996). TIMPs are specific inhibitors that bind MMPs in a 1:1 stoichiometry (Visse & Nagase 2003). All TIMPs (TIMP-1, -2, -3 and -4) are capable of inhibiting all MMPs, with the exception that TIMP-1 is a poor inhibitor of MMP-19 and most of the MT-MMPs (Baker *et al.* 2002). TIMP-3 appears to be a more potent inhibitor of MMP-9 than other TIMPs (Sternlicht & Werb 2001). Although TIMP-2 inhibits MMP-2 in high concentrations, it has an important role in activating proMMP-2 in a complex with MT1-MMP (Strongin *et al.* 1995). MT1-MMP is the most efficient MMP-2 activator; however, also MT3-MMP, and in some species MT2-MMP, is able to activate MMP-2 (Sternlicht & Werb 2001). At first, TIMP-2 binds to MT-MMP and at the same time acts as a receptor for proMMP-2. Then, another MT-MMP cleaves and activates the bound proMMP-2. Fully active form of MMP-2 is achieved by removal of the residual portion of the propeptide by another MMP-2 molecule. (Sternlicht & Werb 2001) TIMP-4 can prevent the activation by displacing TIMP-2 (Hernandez-Barrantes 2002).

In addition to MMP-inhibiting activities, TIMPs have many important biological functions. TIMPs can promote or inhibit cell growth, depending on the cell type and inductor (Baker *et al.* 2002, Visse & Nagase 2003). While TIMP-1 and TIMP-2 have antiapoptotic activity, TIMP-3 is proapoptotic (Baker *et al.* 2002). Great clinical interest is focused on the reduction of tumor growth by over expression of TIMP-1, TIMP-2 and TIMP-3, although their use in therapy has so far been disappointing (Whittaker *et al.* 1999, Baker *et al.* 2002). Although TIMPs inhibit the growth of some cancer cells *in vivo*, upregulation of TIMP-1 is often associated with a poor prognosis, since TIMP-1 may even promote tumor growth in an MMP-dependent or -independent manner (Sternlicht & Werb 2001).

The TIMPs expressed in skeletal muscle are TIMP-1, TIMP-2 and TIMP-3 (Singh *et al.* 2000, Balcerzak et al 2001). TIMP-4 appears to be cardiac-specific (Li *et al.* 2000), and it has not been found in skeletal muscle.

2.5.1.2 MMP catabolism

While TIMPs inhibit MMPs in a reversible manner, other factors are responsible for the irreversible clearance of MMPs from plasma and tissues (Sternlicht & Werb 2001). Most MMPs can be trapped by α2-macroglobulin. The α2-macroglobulin/MMP complex disables the MMP activity, and eventually the complex is endocytosed and cleared. Thrombospodin-2 (TS2) binds both latent and active MMP-2 and the TS2/MMP comlex is bound and endocytosed by low-density lipoprotein receptor-related protein LPR. LPR is also required for the internalization and degradation of MMP-13 after binding to an MMP-13 specific receptor present on various cell types. (Sternlicht & Werb 2001)

2.5.2 Other forms of collagen degradation

Degradation prior to secretion occurs in the Golgi apparatus (Laurent 1987). This type of degradation is suggested to represent basal turnover, while intracellular degradation in lysosomes occurs when the rate of degradation increases due to the production of defective collagen (Laurent 1987). If the collagen is secreted, degradation may occur either before or after the incorporation of the collagen molecules into a fibril. Phagocytosis of collagen fibrils by fibroblasts seems to be continuous process in the remodeling of the ECM (Everts *et al.* 1996). After phagocytosis, collagen is digested in lysosomes by cysteine proteinases such as cathepsin B and/or L (Everts *et al.* 1996). Phagocytosis and intracellular digestion is modulated by cytokines and growth factors, including TNFα, TNFβ, IL-1α and TGFβ (van der Zee *et al.* 1995, Chou *et al.* 1996, Everts *et al.* 1996).

2.6 Adaptation of connective tissue in skeletal muscle

2.6.1 Effects of physical activity

Short- and long-term endurance training (Suominen & Heikkinen 1975, Suominen *et al.* 1977, Kovanen *et al.* 1980, Takala *et al.* 1983, Kovanen & Suominen 1989), downhill running (Han *et al.* 1999a, Koskinen *et al.* 2001) and forced lengthening contractions (Koskinen *et al.* 2002) seem to enhance the activity of enzymes participating in the

posttranslational modifications of collagens in skeletal muscles (Table 2). Also the mRNA levels of type I, III and IV collagens are increased after downhill running (Han *et al.* 1999a, Koskinen *et al.* 2001) and forced lengthening contractions (Koskinen *et al.* 2002). The effects of forced lengthening contractions on type IV collagen concentration vary in different muscles from increased to decreased (Koskinen *et al.* 2002). After downhill running, type IV collagen concentration is increased (Koskinen *et al.* 2001). The gene expression and quantity of (pro)MMP-2 are increased after downhill running (Koskinen *et al.* 2001) and forced lengthening contractions (Koskinen *et al.* 2002), the latter also causing increase in the expression of proMMP-9. While MMP-2 expression is increased, the expression of its inhibitor, TIMP-2, is also upregulated after downhill running (Koskinen *et al.* 2001) and forced lengthening contractions (Koskinen *et al.* 2002). The quantity of TIMP-1 is not changed in these exercise models, although its mRNA level is increased (Koskinen *et al.* 2001 and 2002).

2.6.2 Effects of disuse

Immobilization, suspension and hypogravity are commonly used as experimental models of disuse. Disuse induces muscle atrophy by causing decreased protein synthesis (Goldspink *et al.* 1986), increased protein degradation (Goldspink *et al.* 1986) and apoptosis (Allen *et al.* 1997). Reduction in muscle cell size is accompanied with enhanced volume of connective tissue first observed in perimysium and after one week of continuous disuse also in endomysium (Williams & Goldspink 1984, Sancesario *et al.* 1992). The metabolism and function of muscle cells are impaired not only due to atrophic changes, but also due to decreased capillary density (Jozsa *et al.* 1990) and increased volume of connective tissue surrounding the remaining capillaries, separating them from muscle cells (Järvinen *et al.* 2002). Immobilization in shortened position causes transformation of muscle cells towards type II, fast cells (Hurme *et al.* 1990, Laurila *et al.* 1991, Goldspink *et al.* 1992).

Although the amount of connective tissue increases during immobilization, the gene expression of type I and III collagens decreases during the first three days of immobilization (Table 2.) (Han *et al.* 1999b). Apparently the decrease is transient, thus Miller and co-workers (2001) did not observe any effect on the expression of type I and III collagens in soleus muscle during hindlimb unloading for 1, 14 and 28 days. The activities of prolyl 4-hydroxylase (P 4-H) and galactosylhydroxylysyl glucosyltransferase (GGT) decrease from the first three days of immobilization up to at least three weeks (Savolainen *et al.* 1987 and 1988, Karpakka *et al.* 1991, Han *et al.* 1999b), suggesting decreased collagen biosynthesis during that time. Total muscular collagen content does not change during the first four weeks of disuse (Savolainen *et al.* 1987, Karpakka *et al.* 1991, Han *et al.*1999, Miller *et al.* 2001). The proportion of type I collagen out of type I and III does not change during the first week of immobilization (Han *et al.* 1999b), however hindlimb unloading for two to four weeks causes the proportion of type I collagen to decrease (Miller *et al.* 2001). The effects of disuse on type IV collagen have not been studied previously.

Disuse is known to cause increased breakdown of muscular proteins (Goldspink *et al.* 1986). However, very little is known of the effects of disuse on degradation of collagens (Table 2). Only one previous study has been reported where MMPs are measured. In that study, expression of both MMP-2 and MMP-9 is increased after 30 days of immobilization in rat muscles (Reznick *et al.* 2003), suggesting enhanced degradation of collagens especially in the basement membrane. The increased amount of MMP-9 is probably associated with the increased number of leucocytes in the muscle tissue. TIMP-1 quantity is also increased after 30 days of immobilization (Reznick *et al.* 2003). The observed degenerative changes may contribute to the above mentioned decrease in capillary density during immobilization.

The length of the muscles during immobilization is known to have profound effects on the time course and extent of muscle atrophy. Immobilizing muscles in lengthened position delays the onset of atrophy (Booth 1977, Hurme *et al.* 1990) and may even cause increased protein synthesis (Goldspink *et al.* 1986) and muscle hypertrophy (Booth 1977). Stretch itself is known to cause repressed expression of fast myosin heavy chain genes and increased expression of slow myosin HC gene (Goldspink *et al.* 1992). Immobilizing fast muscle in lengthened position induces a shift of fast glycolytic type IIb fibers towards more oxidative IIa forms (Laurila *et al.* 1991). Immobilizing plantarflexors in lengthened position prevents the decrease in activities of P 4-H and GGT after three weeks, suggesting prevention of decrease in collagen biosynthesis (Savolainen *et al.* 1988).

2.6.3 Effects of glucocorticoids

Glucocorticoids are potent inhibitors of collagen synthesis and are therefore used clinically in the therapy of fibrotic conditions of the liver (Tanner & Powell 1979), lung (Turner-Warwick *et al.* 1980) and skin (Griffiths 1966). Glucocorticoids inhibit collagen synthesis by reducing mRNA levels of type I and III collagens (Oikarinen *et al.* 1983, Graham *et al.* 1995), and activity of enzymes participating in the posttranslational modifications of collagens (Oikarinen *et al.* 1986). Systemic glucocorticoids in doses usually employed in clinical practice decrease collagen synthesis in human skin (Autio *et al.*1994). Dexamethasone also decreases the gene expression and secretion of type IV collagen from the cultured human fibrosarcoma cells (Oikarinen *et al.* 1987). No previous results have been published on the effects of glucocorticoids on collagens in skeletal muscle.

Large doses of glucocorticoids, often used in severe asthma, can cause acute corticosteroid myopathy (ACM). A typical finding in ACM is severe muscle weakness, even quadriplegia, with necrotic changes in muscle tissue (Hanson *et al.* 1997). Simultaneous muscle disuse may predispose to ACM, because it increases the number of cytoplasmic corticosteroid receptors (DuBois & Almon 1980). Glucocorticoid overproduction and overuse cause muscle atrophy and loss of muscle function (Seene & Viru 1982), particularly destruction of myofilaments (Seene *et al.* 1988). The overall protein synthesis in skeletal muscle is inhibited by glucocorticoids (Rannels *et al.* 1980,

Long *et al.* 2001) and even single-dose glucocorticoid treatment rapidly increases the gene expression and enzymatic activity of glutamine synthetase in skeletal muscle, indicating induced protein catabolism (McKay *et al.* 1997).

Glucocorticoids, as anti-inflammatory agents, decrease MMP levels in fibroblasts (Morin *et al.* 1999), magrophages (Shapiro *et al.* 1991), malignant cells (Kylmäniemi *et al.* 1996) and injured spinal cord (Xu *et al.* 2001). They also decrease TIMP-1 activity in magrophages (Shapiro *et al.* 1991). Decreased MMP synthesis caused by glucocorticoids seems to be mediated by the inhibition of two major inflammatory transcription factors: nuclear factor κB (NF- κB) and activator protein (AP-1) (Xu *et al.* 2001). Tumor necrosis factor α (TNF-α), a proinflammatory agent regulated by NF- κB and a stimulator of MMP expression, has been found to be suppressed by glucocorticoid administration in fibroblasts (Morin *et al.* 1999). The effects of glucocorticoids on MMPs or TIMPs have not been previously studied in skeletal muscle.

The effect of physical activity on the myopathy caused by glucocorticoids is contradictory. Endurance running can either prevent (Hickson & Davis 1981, Seene & Viru 1982, Falduto *et al.* 1992), fail to prevent (Fimbel *et al.* 1993) or even pronounce (Lieu *et al.* 1993) the effects of glucocorticoids on muscle contractile component. While weight lifting prevents the glucocorticoid-induced atrophy (Gardiner *et al.* 1980), sprint training is not able to counteract the effects of corticoids (Fimbel *et al.* 1993). Repetitive stretch-relaxation of cultured myofibers partly prevents the decrease in protein synthesis induced by glucocorticoids (Chromiak & Vandenburg 1992).

2.6.4 Effects of neuromuscular diseases

Inflammatory myopathies are characterized by chronic inflammation, loss of muscle fibers and fibrosis (Dalakas 2001). In polymyositis, autoinvasive T-cells destroy muscle fibers that express specific endogenous muscle peptides or virus genomes associated with the major histocompatibility complex (MHC) antigens (Dalakas 2001). Cell death is caused by osmotic cell lysis (Goebels *et al.* 1996) without any signs of apoptosis (Dalakas 2001). Increased collagen synthesis most likely has a key role in the progressive fibrosis (Table 2) (Myllylä *et al.* 1982). The accumulation of collagen in the ECM in inflammatory myopathies (Foidart *et al.* 1981, Peltonen *et al.* 1982) is mediated by increased gene expression and activities of enzymes participating in the posttranslational modifications of collagens (Myllylä *et al.* 1982). Increased gene expression and/or muscular activities of MMP-1 (Kieseier *et al.* 2001), MMP-2 (Choi & Dalakas 2000, Schoser *et al.* 2002), MMP-7 (Schoser *et al.* 2002) and MMP-9 (Choi & Dalakas 2000, Kieseier *et al.* 2001, Schoser *et al.* 2002) are observed during myositis. Various chemokines and growth factors are upregulated in inflammatory myopathies, inducing the expression of MMPs (Dalakas 2001). MMPs participate in the inflammatory process by disrupting the endothelial basement membranes and ECM, thus preparing the way for hematogenous cells (Dalakas 2001). However, despite the enhanced degradation capillary basement membranes become thick and multilaminated as a response to chronic inflammation (Vlodavsky *et al.* 1999).

Peripheral neuropathy is usually caused by repetitive activities, pressure on a nerve, nutritional deficiency, some illnesses (e.g. diabetes), medication or chemicals (e.g. alcohol). Chronic and acute inflammatory demyelinating polyneuropathies, and nonsystemic vasculitic neuropathy are linked to autoimmune responses (Moore 1989, Kieseier *et al.* 2002). Peripheral neuropathies impair the function of nerves, for example by demyelination and/or axon degeneration (Thomas 1999, Kieseier *et al.* 2001). Although neurogenic atrophy and fiber type grouping occur in muscles affected with peripheral neuropathy, loss of inflammation and necrotic changes in muscle is the most distinguishing factor between inflammatory myopathies and neuropathies (Schoser *et al.* 2001). Minor evidence of apoptosis appears in neuropathic muscles (Schoser *et al.* 2001). Studies on the effects of polyneuropathy on connective tissue have focused on nerves. These studies suggest collagen deposition inside the nerves (Bradley *et al.* 2000) and changes in the neuronal basal lamina during diabetic polyneuropathy (King *et al.* 1989), although deposition of type IV collagen seems not to be involved in the early stages (Williams *et al.* 2000). MMP-2 and MMP-9 expression is enhanced in the nerves with chronic inflammatory demyelinating polyneuropathies and nonsystemic vasculitic neuropathy (Leppert *et al.* 1999) The very few studies concerning muscular connective tissue suggest that polyneuropathy is characterized by slight changes in collagen synthesis without accumulation of collagen in affected skeletal muscles (Myllylä *et al.* 1982, Peltonen *et al.* 1982). In denervated human muscles, the immunohistological staining of MMP-9 is increased in chronic axonal neuropathy (Schoser & Blottner 1999).

Table 2. The effects of immobilization, exercise, neuromuscular diseases and glucocorticoids on collagen synthesis and the activities of MMPs and TIMPs

Study	Collagen			Expression				
	mRNAs	synthesis	coll IV	MMP-1	MMP-2	MMP-9	TIMP-1	TIMP-2
Immobilization								
Han et al. 1999	↓	↓						
Savolainen *et al.* 1987, 1988		↓						
Karpakka *et al.* 1991		↓						
Miller *et al.* 2001	—							
Reznick *et al.* 2003					↑	↑		
Exercise								
Suominen & Heikkinen 1975		↑						
Suominen *et al.* 1977		↑						
Kovanen *et al.* 1980		↑						
Takala *et al.* 1983		↑						
Kovanen & Suominen 1989		↑						
Han *et al.* 1999	↑	↑						
Koskinen *et al.* 2001	↑	↑	↑ ↓		↑		—	↑
Koskinen *et al.* 2002	↑	↑	↑		↑	↑	—	↑
Myositis								
Myllylä *et al.* 1982	↑	↑						
Choi & Dalakas 2000					↑	↑		
Kieseier *et al.* 2001				↑	—	↑	—	—
Schoser *et al.* 2002					↑	↑		
Polyneuropathy								
Myllylä *et al.* 1982	—	↑						
Glucocorticoids								

— = no change to control; ↑ = increased; ↓ = decreased; empty space = not studied.

3 Purpose of the study

The purpose of the present study was to investigate collagen turnover in skeletal muscle within conditions causing muscle atrophy and loss of muscle function. In particular, type IV collagen and proteins regulating its degradation were studied during immobilization, glucocorticoid administration and neuromuscular diseases. In addition, fibrillar type I and III collagens were studied during immobilization and neuromuscular diseases. To investigate the role of muscle length during immobilization, the ankle of a rat was immobilized to plantarflexion or dorsiflexion, thus causing the leg muscles to be either lengthened or shortened. Two types of exercise were used to investigate their possible role in preventing glucocorticoid-induced changes. More specifically, the purpose was to study

1. synthesis of type IV collagen in skeletal muscle during immobilization, dexamethasone treatment and polyneuropathy (II, III, IV)
2. regulation of degradation of type IV collagen in skeletal muscle by measuring the expression of MMP-2 during immobilization, dexamethasone treatment and polyneuropathy (II, III, IV), and the expression of TIMP-2 during immobilization and dexamethasone treatment (II, III)
3. total collagen biosynthesis in skeletal muscle during immobilization (I)
4. synthesis of type I and III collagens in skeletal muscle during immobilization and neuromuscular diseases (I, IV)

4 Material and methods

4.1 Animals (I, II, III)

The animals were young adult Sprague-Dawley male (studies I and II) or female (III) rats. The animals were housed in individual (I and II) or group (III) cages under constant temperature (22 ºC), humidity (40 %) and light-dark cycle (light between 6:00 AM and 6:00 PM) conditions. The animals were maintained on a standard rodent food diet with water *ad libitum*. Casting (I and II) was performed under neuroleptic anesthesia (fentanyl citrate 0.0315 mg/ml, fluanisone 10 mg/ml; Hypnorm, Janssen Pharmaceutica; Oxford, UK), 0.5 ml/kg body weight. Control animals were also anesthetized at the beginning of the experimental period. Before the beginning of the experiment, the rats were randomized into six different experimental groups and a control group, 8-9 animals in each group. In study III, rats were treated daily with intraperitoneal injections of dexamethasone, 1 mg / kg body weight, or corresponding volume of salt solution, 0.15 M NaCl. Rats were divided to a total of 12 groups, each containing 6 animals. Eight groups in total were engaged to either endurance or uphill running exercise on a treadmill. Four of these exercise groups were simultaneously treated with dexamethasone. Four groups remained sedentary with or without dexamethasone treatment. At the end of the experimental period, rats were anesthetized (I and II) or sedated with carbon dioxide (III) and sacrificed with decapitation. All treatment of the animals was in accordance with the European Convention for the Protection of the Vertebrate Animals Used for Experimental and Other Scientific Purposes and were controlled by the Committee of Laboratory Animal Experiments, University of Jyväskylä.

4.2 Human subjects (IV)

The study consisted of 38 patients with a neuromuscular disease (21 men and 17 women aged 21 to 74 years) and 34 control subjects (19 men and 15 women, aged 18 to 68 years). The patient group comprised seven patients with polymyositis, 13 patients with

myopathy (two sarcoidosis, two systemic lupus and nine unspecific myopathy), and 18 patients with peripheral neuropathy, among whom seven were diagnosed as axonal, four as demyelinating, and seven as mixed type of neuropathy. Eight of the neuropathies were caused by diabetes mellitus, four by alcohol and one by paraproteinemia, and five were diagnosed as an idiopathic peripheral neuropathy. The diagnosis was based on clinical and histological criteria and the findings of the electroneuromyographic examination. None of the control subjects had diseases or medication known to affect the human neuromuscular system, and none of the subjects had received immunomodulatory therapy prior to muscle biopsy and blood sampling. The study was approved by the Ethics Committee of the Medical Faculty, University of Oulu, and informed consent was obtained from each subject.

4.3 Immobilization (I, II)

The right hind-limb of a total of 53 experimental animals was immobilized with a plaster of Paris, so that the ankle joint was in full plantarflexion (150-160º ankle between foot and leg) or in dorsiflexion (30-40º ankle). The same casting technique has been used previously by Laurila et al. (1991). The casts were inspected daily and repaired if necessary. The length of the immobilization period was 1, 3 or 7 days, previously used by Han et al. (1999). A control group of a total of 9 animals included 3 sedentary animals from each observation point.

4.4 Glucocorticoid treatment and exercise (III)

Four groups with 6 rats in each group had either dexamethasone or NaCl treatment for 3 or 10 days, and remained sedentary without exercise (Fig. 4). Eight groups with 6 rats in each were engaged to exercise for 3 or 10 days with or without simultaneous dexamethasone treatment. 10 days glucocorticoid treatment has previously been used by Seene *et al.* (1988) and Lieu et al. (1993). 3 days treatment has been found to cause reduction in muscle mass (Falduto *et al.* 1992) and was therefore chosen for the shorter experimental period. The animals were familiarized with the treadmill before the beginning of the experiment. For endurance running groups, the duration of exercise was progressively increased from 15 minutes to a maximum of 45 minutes while the velocity of treadmill was kept constant at 20 meters/min. Total duration of exercise was 60 min for animals treated for 3 days, and 5 hours 15 min for animals treated for 10 days. In the uphill running groups, the animals ran 5-15 times 30 s spurts separated by two-minute breaks. Treadmill velocity was 35 m/min during the first 3 days after which it was kept constant at 40 m/min. The treadmill inclination was 5º. Duration of daily exercise was progressively increased from 2 min 30 s to a maximum of 7 min 30 s during 10 days. Total duration of exercise was 10 min 30 s for rats exercising 3 days, and 51 min 30 s for rats exercising 10 days.

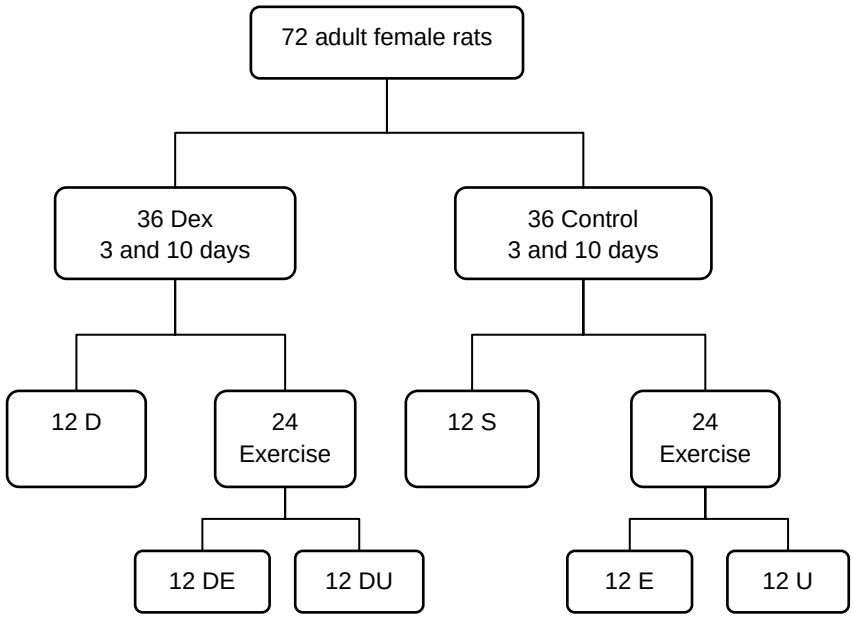

Fig. 4. Dexamethasone treatment and exercise in study III. The number of animals in each group is presented inside the boxes. All groups have two subgroups with 6 animals in each for the 3- and 10-day treatments. D = dexamethasone; S = sedentary; E = endurance running; U = uphill running.

4.5 Muscle samples

In the immobilization experiment (I and II), soleus (SOL), extensor digitorum longus (EDL), gastrocnemius (GM) and tibialis anterior (TA) muscles from the right leg were excised and weighed. GM and TA were cut in half and the proximal parts were used for protein analyses and distal parts for mRNA analyses. Due to the small size of SOL and EDL, muscles from 4 rats were used for protein analyses, whereas the muscles from 5 rats were used for mRNA analyses. Small slice was cut for microscopy from the center of each muscle. TA was left out of study II due to lack of response to immobilization observed in study I. In the glucocorticoid experiment (III), EDL, SOL and TA muscles from both legs of control and experimental animals were excised and weighed. Muscles from the right leg were used for mRNA analyses, and from the left leg for protein analyses. TA muscle was cut in two parts, from which distal parts were used. Muscle biopsies from neuromuscular patients (IV) were obtained by concotomy in local anesthesia from deltoideus, TA, rectus femoris and vastus lateralis muscles. Part of the biopsies was used for histology and immunohistochemistry, the other parts for mRNA and protein analyses. mRNA analyses were the primary analyses. Protein analyses were

conducted only if the size of the specimen was sufficient. Summary of the methods used in different studies is presented in Table 3.

The samples for mRNA and protein analyses were first immersed in liquid nitrogen and then stored at -70 ºC for further analysis. For immunohistochemistry, the specimen was mounted in embedding medium (OCT Compound, Tissue-Tek®, Sakura Finetek, Zoeterwoude, The Netherlands) on a cork disk and rapidly frozen in isopenthane cooled to -160 ºC in liquid nitrogen.

4.6 mRNA analyses

For total RNA isolation, muscle samples were homogenized with an Ultra-Turrax homogenizer in Trizol (Life Tecnologies, Painsley, Scotland, UK). Other steps were performed as described in the manufacturer's protocol (Life Tecnologies 1995). The purity and concentration of total RNA was assessed spectrophotometrically. For Northern blotting, 30 µg of total RNA was denatured in loading buffer, electrophoresed in a 1 % agarose/formaldehyde gel, and transferred to a nylon membrane (GeneScreen Plus, Biotecnology Systems, Boston, USA) with a standard procedure (Chomczynski & Mackey 1994). For slot blotting, 20 µg of total RNA was spotted on a nylon membrane using a vacuum filtration manifold (Minifold II; Schleicher and Schuell, Dassel, Germany) (Maniatis *et al.* 1982). All the membranes were incubated in 0.05 N NaOH for 5 min to bind the RNA to the membrane. Prehybridization was carried out in a solution containing 5 x SSC, 5 x Denhart's solution, 50 % formamide, ssDNA 100 µg/ml, 50 mM sodium phosphate pH 6.8, 10 % dextran sulphate and 1 % SDS for 2 hrs at 42 ºC. The RNA-cDNA hybridization was performed for 24 hrs at 42 ºC using the solution containing the same components as the prehybridization solution and [^{32}P]-labeled cDNA probe labeled with a Ready-To-Go -DNA Labeling Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). After hybridization, the membranes were washed at 65 ºC with 2 x SSC + 2 % SDS solution. The membranes were exposed to Kodak X-Omat film at -70ºC. Bands were analysed using densitometry (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, CA, USA). The signal obtained by hybridization with a 24 mer oligonucleotide for 18S ribosomal RNA was used to normalize RNA loading/transfer amount.

4.7 Biochemical assays

For biochemical measurements, samples were homogenized with an Ultra-Turrax homogenizer in two 10 s bursts in a cold solution containing 0.2 M NaCl, 0.1 % Triton X-100 and 0.02 M Tris-HCl buffer, pH adjusted to 7.5 at 4 ºC. The homogenates were centrifuged (15,000 g for 30 min at 4 ºC), and the pellets were used for the radioimmunoassay of 7-S collagen, analyses of hydroxyproline (Hyp) and measurement of the proportions of type I and III collagens. The supernatants were used for the assays

of soluble protein concentration, prolyl 4-hydrozylase (P4-H), zymography and reverse zymography. Soluble protein concentration was measured by a commercial BioRad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

4.7.1 Prolyl 4-hydroxylase activity (I)

The activity of P4-H, which is one of the enzymes regulating the posttranslational modifications of the collagen polypeptide chains, was measured by the labeled hydroxyproline formed from peptide-bound prolyl residues of unhydroxylated labeled protocollagen substrate (Kivirikko & Myllylä 1982). Enzymatic activity was assayed in final volume of 1 ml containing 70 µl supernatans from centrifuged muscle homogenates, 7 µl 0.1 % (w/v) soybean trypsin inhibitor, 7 µl 0.5 mM dithiothreitol, 50 000 dpm proline-labeled protocollagen-^{14}C, 0.08 mM $FeSO_4$, 0.5 mM α-ketoglutarate, 2 mM ascorbic acid, 0.002 % (w/v) bovine serum albumin, and 50 mM tris-HCl buffer, pH 7.8. Test tubes were incubated at 37 °C for one hour, and the reaction was stopped by addition of 1 ml concentrated HCl. The samples were hydrolyzed overnight at 120 °C. After evaporation, the residues were dissolved in 4 ml dH_2O and the total ^{14}C-contents were measured with a liquid scintillation counter.

4.7.2 Proportions of type I and III collagens (I)

The muscle pellets were resuspended in 2 % SDS in order to remove noncollagen proteins. The remaining residues were suspended in 1 ml of 70 % formic acid and digested by adding 5 µg cyanogens bromide per 1 mg muscle wet weight. The samples were digested at 35 °C for 4 hours. The reaction was stopped and cyanogens bromide was removed by diluting with several volumes of distilled water and evaporation (Laurent *et al.* 1981). The remaining residues were resuspended in water and divided into hydroxyproline concentration measurement and cyanogen bromide peptide electrophoresis. The samples were dissolved to a final hydroxyproline concentration of 1 µg/µl in a solution containing 2 % SDS, 6.25 mM Tris-HCl, 10 % glycerol, 5 % (v/v) β-mercaptoethanol, and 0.05 % (w/v) bromophenol blue at 95 °C for 4 minutes. The samples were loaded on 8-20 % linear gradient SDS polyacrylamide gels with 4 % stacking gels and electrophoresed overnight at 70 V. After fixation, gels were stained with Coomassie brilliant blue. For type I collagen quantitative determination the density (Molecular Dynamics) of the α_1 (I) CB8 peptide band, and for type III collagen the density of the α_1 (III) CB5 peptide band was measured and compared with the respective CB peptides of digested type I (rat tail, C8897, Sigma, St. Louis, MO, USA) and III (recombinant human, generously provided by Dr. K. Kivirikko, Department of Medical Biochemistry, University of Oulu, Finland) standards (Light 1982).

4.7.3 Total collagen concentration (I, II)

Hydroxyproline concentration represents total collagen concentration, and was measured by the method of Kivirikko *et al.* (1967). Sample supernatant was mixed with concentrated HCl and incubated at 110 °C for 16 hours. Hydroxyproline was oxidated with chloramine T solution to pyrrole, which was extracted into toluene. Ehrlich's reagent was mixed with toluene for color reaction. Absorbance was measured at 560 nm using spectrophotometer (UV-160, Shimazu, Osaka, Japan).

4.7.4 Radioimmunoassay for 7-S (II, III, IV)

7-S collagen, which is the N-terminal domain for type IV collagen, was measured to estimate the concentration of type IV collagen in muscles. For solubilization of 7-S collagen, the pellet of the muscle sample homogenate was suspended in 0.2 M ammoniumbicarbonate. The samples were digested first with bacterial collagenase (CLSPA, Worthington, Freehold, New Jersey, USA; 200 or 60 µg/100 mg of tissue in 0.2 M ammoniumbicarbonate) for 24 hrs at room temperature followed by trypsin treatment (TPCK-treated, Sigma; 100 µg/100 mg of tissue in 0.2 M ammoniumbicarbonate) for 12 hrs at room temperature. Finally, soybean trypsin inhibitor (Sigma; 200 µg/100 mg of tissue in 0.2 M ammoniumbicarbonate) was added and the samples were incubated for 20 min at 37 °C before centrifugation (15,000 g for 30 min at 4 °C). Supernatants were stored at -20ºC until analysed by radioimmunoassay.

The radioimmunoassay for 7-S collagen was performed as an equilibrium type of inhibition assay described by Risteli *et al.* (1981a). ^{125}I-labeled and non-labeled (inhibitor) antigens in various dilutions were first mixed, and then antiserum capable of binding 50 % of labeled antigen was added. After incubation, free and bound antigens were separated by precipitation with goat antiserum to rabbit IgG. Concentration of bound sample antigen (inhibitor) was calculated from the inhibition curve. 7-S collagen is resistant to degradation of proteases, and its stable antigenic structure makes it easier to detect by immunochemical analysis than the intact type IV collagen (Risteli *et al.* 1981b). To estimate the concentration of type IV collagen it was assumed that 7-S collagen comprises about 20 % of the mass of type IV collagen (Risteli *et al.* 1980).

4.7.5 Zymography (II, III, IV)

Zymography was used for quantification of gelatinolytic enzymes MMP-2 and MMP-9, and was carried out with minor modification of the method of Kleiner & Stetler-Stevenson (1994). SDS polyacrylamide gels (11 %) containing 1 mg/ml gelatin were overlaid with 4 % stacking gels. Sample supernatants were mixed with 1:1 volume of a sample buffer consisting of 50 mM Tris, pH 6.8, 2 % SDS, 20 % glycerol, and 0.03 %

bromphenol blue. After loading the samples into the wells of a gel, electrophoresis was carried out at 80V until the visible dye had reached the bottom of the gel. Gels were then incubated first in a solution containing 2 % Tween 80 and 50 mM Tris, pH 7.5, and then twice in a solution containing additionally 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ at 37 °C in order to remove SDS from the gels and activate gelatinases. Gelatinase activity was revealed by negative staining with Coomassie Brilliant blue and quantified by densitometry (Personal Densitometer SI). Values of optical density were used for statistical analyses. For identification of MMPs, each gel contained a lane of purified proMMP-2 and proMMP-9 standard (Diabor, Oulu, Finland).

4.7.6 Reverse zymography (II, III)

TIMP-1 and TIMP-2 proteins were analysed with reverse zymography as described by Oliver *et al.* (1997). In reverse zymography, gelatin and gelatinase are incorporated directly into acrylamide gels and inhibitory activity of TIMPs results in dark blue areas after staining. 12 % SDS polyacrylamide gels contained gelatin and proMMP-2 in concentrations that were optimized for different muscles with test gels made from some of the samples. 4 % stacking gel was used. Sample supernatants were mixed with 1:5 volume of sample buffer containing 40 mM Tris, pH 6.8, 5 % SDS, 20 % glycerol, and 0.03 % bromphenol blue, and electrophoresed until the visible dye had reached the bottom of the gel. To remove SDS and activate gelatinases, gels were incubated first in 2.5 % Triton X-100 and then in solution containing 50 mM Tris, pH 7.5, 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ at 37 °C. Gels were stained with Coomassie Brilliant blue. The inhibitory activity of TIMPs showed in a dark blue band on a clear background. The quantity of TIMPs was quantified by densitometry (Personal Densitometer SI). For identification, purified TIMP-1 and TIMP-2 (Diabor) was used in each gel.

Table 3. Main analyses used in the studies.

	mRNA	Protein	Method for protein measurement	Original article
P 4-H	X	X	^{14}C labeled hydroxyproline	I
Hydroxyproline		X	Spectrophotometry	I, II
Collagen I	X	X	Cyanogen bromide electrophoresis	I
Collagen III	X	X	Cyanogen bromide electrophoresis	I
Collagen IV	X	X	7-S RIA	II, III, IV
(pro)MMP-2	X	X	Gelatin zymography	II, III, IV
(pro)MMP-9		X	Gelatin zymography	IV
TIMP-1		X	Reverse zymography	III
TIMP-2		X	Reverse zymography	II, III

4.8 Immunohistochemistry (IV)

Muscle biopsy specimens were oriented transversely to muscle fibers under stereomicroscope, mounted in embedding medium (OCT Compound, Tissue-Tek®) on a cork disk, and rapidly frozen in isopenthane cooled to around -130-160 °C in liquid nitrogen. 10 μm frozen sections were cut using cryomicrotome. Hematoxylin-eosin (HE), modified Gomori trichrome, NADH-tetrazolium reductase, and ATPase at pH 9.4, 4.6 and 4.3 stainings were made for diagnostics. With NADH and ATPase stainings the muscle cell types can be specified, which is important e.g. in the diagnosis of denervation and neuromuscular diseases affecting only specific cell types. Gomori trichrome staining reveals abnormal mitochondria often seen in various diseases and vacuoles typical for inclusion body myositis. Part of the biopsy specimens was embedded in paraffin after formalin fixation. For immunohistochemistry, 5 μm sections were deparaffinized, rehydrated and PBS-washed. Immunostaining was performed in an automatic staining machine using a labeled streptavidin-biotin immunoenzymatic antigen detection system (UltraVision Large Volume Detection System Anti-Polyvalent, HRP, Labvision Corporation, Fremont, CA, USA) according to the manufacturer's instructions. 3,3-Diaminobenzidine tetrahydrochloride (DAB) was used as chromogen. The following antibodies were used: rabbit polyclonal IgG against human (1) PINP, (2) PIIINP and (3) IIINTP (dilutions 1:10 000, 1:4000 and 1:1000, respectively; all kind gifts from Dr. J. Melkko, Department of Pathology, University of Oulu, Finland); mouse monoclonal IgG against human (4) type IV collagen (dilution 1:100; Dako A/S, Glostrup, Denmark) and (5) MMP-2 (dilution 1:200; Chemicon, Temecula, CA, USA); (6) rabbit polyclonal IgG against human MMP-9 (dilution 1:100; a kind gift from Dr. L. Kjeldsen, Department of Hematology, University Hospital, Rigshospitalet, Copenhagen, Denmark). For negative controls, the primary antibody was substituted with the buffer (PBS) or with non-immune anti-IgG. The staining results were estimated semiquantitatively, using a three-step scale from slightly increased to highly increased.

4.9 Statistical analyses

A nonparametric Mann-Whitney U-test was used for statistical evaluation of the results in studies I and II. Statistical evaluation of the results in study III was performed using analysis of variance. Individual tests between two groups were not performed in study III due to the large number of groups, which would considerably increase the risk of false positive significances. In study IV, one-way analysis of variance and Student's t-test were used for statistical evaluation of the mRNA data, and nonparametric Mann-Whitney U-test for protein data. $P<0.05$ was considered statistically significant.

5 Results

5.1 General responses to immobilization, glucocorticoids and exercise (I, II, III)

The body mass of the rats decreased during the first day of immobilization, but normal weight gain was achieved by the 7th day of immobilization. Dexamethasone treatment decreased the body mass of the rats already during the first three days. After 10 days, the body mass of all dexamethasone-treated animals was much lower compared to rats without steroid treatment. Both endurance and uphill running exercise increased body mass during 10 days, although exercise combined with dexamethasone treatment even enhanced the decrease in body mass during the first three days. The muscle mass of all muscles studied decreased during both immobilization and dexamethasone treatment. During immobilization, muscle mass of SOL and GM were decreased the most, and shortened position further enhanced the decrease. Exercise only slightly increased the mass of SOL, and it had no effect on the weight of other muscles. However, endurance type of exercise prevented the dexamethasone-induced muscle weight loss in EDL and SOL.

Total RNA content was decreased after immobilization first in GM, and then after three days also in SOL. No change in the RNA content was observed in EDL and TA during immobilization. During dexamethasone treatment, a decrease in the RNA content was observed first in SOL and TA, and after 10 days also in EDL. No changes in soluble protein concentration were observed during immobilization and dexamethasone treatment, except for a slight increase in lengthened SOL after 3 days and shortened TA after 7 days of immobilization. After 7 days of immobilization, soluble protein content (mg/muscle) was decreased in shortened SOL.

5.2 Effects of immobilization on collagens in skeletal muscle (I, II)

5.2.1 Total collagen biosynthesis

Specific mRNA level for P4-H α-subunit was decreased in shortened SOL after 7 days of immobilization. No decrease was observed in lengthened SOL or the other muscles, but the total muscular levels of P4-Hα mRNA were decreased in GM after 3 and 7 days and in SOL after 1 and 7 days in both positions and after 3 days in shortened position. Summary of the main results is presented in Table 4.

In shortened SOL and GM, the specific activity of P4-H (activity/mg soluble protein) was decreased after 3 and 7 days of immobilization. In lengthened SOL and GM, a decrease was observed throughout the whole experimental period. P4-H activity was 37 % lower after 3 days and 30 % lower after 7 days in shortened compared to lengthened GM. In TA, the activity was decreased after 3 days of immobilization in both positions. An increase was observed in shortened and lengthened positions of EDL after the first day of immobilization. Total muscular P4-H activity showed similar changes, except in EDL where a decrease was observed only after 3 days in both positions.

The concentration of Hyp (mg/g wet weight) increased during 7 days in shortened SOL, GM, TA and EDL. In SOL, GM and TA, a slightly smaller increase was also observed in lengthened position at the same time, as well as after 3 days in shortened SOL and GM. The increase was 27 % higher in shortened than in lengthened SOL during the first day of immobilization. No change in the total muscular content of Hyp was observed, except for a slight increase in shortened SOL after the first day of immobilization.

5.2.2 Fibrillar collagens

The mRNA level for type I collagen was decreased in shortened SOL after 3 and 7 days, and in shortened GM after 3 days of immobilization, being lower in SOL after 3 and 7 days and GM after 7 days than in the corresponding lengthened muscles. In EDL, a decrease in type I collagen mRNA level was observed after 3 days of immobilization in both positions. Type III collagen mRNA level was decreased after 3 days in shortened SOL, GM and EDL, and in lengthened GM and EDL. After 7 days, a decrease was observed only in GM. No changes in the mRNA levels of either type I or III collagens were observed in TA. The mean proportion of type I collagen out of type I and type III collagens was 65 % in control GM and 62 % in control TA, and these were not changed during immobilization. The proportions of type I and III collagens were not measured from SOL or EDL due to small sample size.

5.2.3 Type IV collagen

The mRNA level for type IV collagen was decreased in shortened SOL and GM throughout the experimental period. A decrease was also observed in lengthened SOL during 3 days and in lengthened GM after 3 and 7 days of immobilization. No change in type IV collagen mRNA level was observed in EDL during immobilization.

A decreased type IV collagen concentration (ng/µg hydroxyproline) was observed in shortened SOL throughout the whole immobilization period. In lengthened SOL, a decrease was observed only after 3 days. In GM, a decrease was observed only in the lengthened position after 3 and 7 days. A decreased type IV collagen concentration was observed in EDL after 1 day (lengthened position), 3 days (both positions) and 7 days (shortened position). When type IV collagen concentration was related to soluble protein concentration, a decrease was observed only in GM after 1 and 3 days.

5.2.4 MMP-2

The proMMP-2 quantity was increased in SOL and EDL after 1 and 3 days of immobilization in both positions. After 7 days, an increase was observed in shortened SOL and lengthened EDL. In GM, an increase was observed only in the shortened position after 3 days, also in comparison to the lengthened position. Active MMP-2 was detectable only in SOL, and an over 100% increase was observed in this muscle after immobilization for 3 and 7 days in shortened position. In lengthened SOL, the MMP-2 quantity remained at control level.

5.2.5 TIMP-2

The TIMP-2 quantity decreased gradually in SOL and GM during 3 and 7 days of immobilization. An increase was observed in lengthened EDL after 7 days compared to control as well as to shortened position. Otherwise, no change in TIMP-2 quantity was observed in EDL.

5.3 Effects of dexamethasone and exercise on type IV collagen synthesis and degradation in skeletal muscle (III)

5.3.1 Type IV collagen

The mRNA level for type IV collagen was decreased in all dexamethasone-treated SOL, EDL and TA muscles after 10 days, with or without exercise. In SOL, exercise increased type IV collagen mRNA level during 3 and 10 days, the increase being most pronounced in the endurance running group after 10 days. Dexamethasone treatment increased type IV collagen concentration during 3 and 10 days in TA and during 10 days in EDL. Summary of the main results is presented in Table 4.

5.3.2 MMP-2

The mRNA level for MMP-2 was decreased after a 10-day dexamethasone treatment only in TA, although a slight decrease in EDL was also observed. Dexamethasone treatment caused a decrease in the proMMP-2 quantity in EDL and TA during 3 days and in all muscles during 10 days. Exercise did not have any effect on proMMP-2 either by itself or with dexamethasone treatment. No active form of MMP-2 was observed in zymography gels.

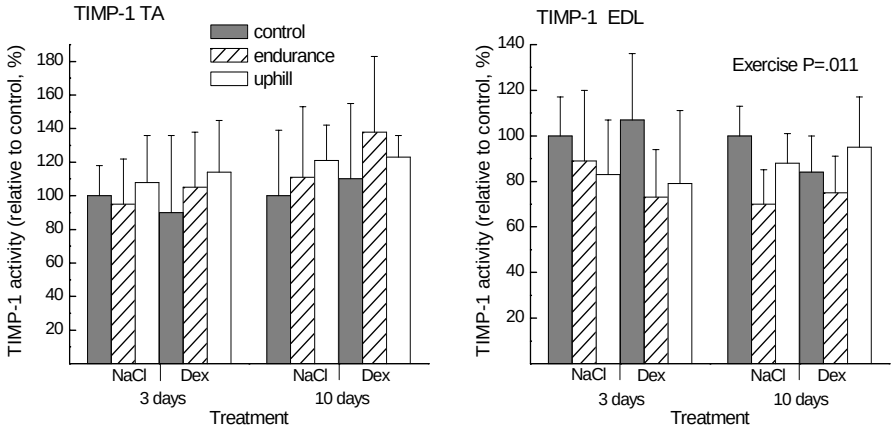

Fig. 5. Activity of TIMP-1 in TA and EDL during dexamethasone treatment and exercise. Dex = dexamethasone.

5.3.3 TIMP-1 and TIMP-2

Dexamethasone treatment did not have effects on TIMP-1 protein in EDL or TA, whereas exercise, especially endurance running, decreased the quantity of TIMP-1 in EDL after 10 days (Fig. 5). The quantity of TIMP-2 was decreased in EDL and TA after 3 and 10 days of dexamethasone treatment. 10-day exercise increased the TIMP-2 activity in TA. In this muscle, uphill running almost entirely prevented the dexamethasone-induced decrease in TIMP-2 quantity. Neither dexamethasone nor exercise had any effect on TIMP-2 in SOL.

5.4 Effects of neuromuscular diseases on collagen and MMPs (IV)

5.4.1 Fibrillar collagens

The mRNA level of type I collagen was 180 % and type III collagen 139 % greater in patients with polymyositis compared to control subjects. Type I and III collagen mRNA levels were also elevated in polyneuropathy (a 66 % and 5 % increase, respectively). Immunohistochemistry revealed increased PINP, PIIINP and IIINTP staining intensities in the perimysium and endomysium of patients with polymyositis and polyneuropathy. In polyneuropathy, PINP was increased mostly in the endomysium, whereas PIIINP and IIINTP were elevated equally in both peri- and endomysium. No differences were observed between the myopathy patients and control subjects in the mRNA levels or immunohistochemical stainings of fibrillar collagens. Summary of the main results is presented in Table 4.

5.4.2 Type IV collagen

The mRNA level of type IV collagen was 118 % greater in polymyositis and 43 % greater in polyneuropathy patients compared to control subjects. The type IV collagen concentration (ng/mg wet weight) was 83 % higher in polyneuropathy (n=4) compared to controls. The immunohistochemical staining intensity for type IV collagen was increased around the muscle cells and capillaries in patients with polymyositis and polyneuropathy. No differences were observed between myopathy patients and control subjects in the mRNA level, protein concentration or staining intensity of type IV collagen.

5.4.3 MMPs

The mRNA level of MMP-1 was 60 % higher in polymyositis compared to controls. The mRNA level of MMP-2 was 168 % elevated in polymyositis and 37 % elevated in polyneuropathy. No difference compared to controls was observed in proMMP-2 quantity (n=5) in muscles of patients with polyneuropathy, but the quantity of proMMP-9 (n=4) was 143 % elevated in this disease. Immunohistochemistry revealed more cells stained for MMP-9 in both polyneuropathy and polymyositis patients compared to control subjects. No differences in any of the MMP markers were observed between myopathy patients and control subjects.

Table 4. Summary of the main findings.

	Immob		Dexamethasone		Exercise	Neuropathy	Myositis
	Short	Length	Sedent	Exerc			
P 4-H							
mRNA	↓↓	↓					
Activity	↓↓	↓					
Hyp	↑	—					
Coll I							
mRNA	↓	-/↓				↑↑	↑↑↑
PINP						↑↑	↑↑↑
Coll III							
mRNA	↓↓	↓				↑↑	↑↑↑
PIIINP						↑↑	↑↑↑
Coll IV							
mRNA	↓↓	↓	↓↓↓	↓↓↓	-/↑↑	↑↑	↑↑↑
Conc.	↓↓	-/↓↓	-/↑	-/↑	—	↑↑	
MMP-2							
mRNA	↑↑	↑	-/↓	-/↓	—	↑	↑↑↑
Protein	↑↑	-/↑	↓↓	↓↓	—	—	
MMP-9						↑↑	
TIMP-1			—	—	-/↓		
TIMP-2	↓↓	↓↓	↓↓↓	↓↓	-/↑		

Immob = immobilized; Short = shortened; Length = lengthened; Hyp = hydroxyproline; Conc = concentration; — = no change to control; ↑ or ↓ = slightly increased of decreased; ↑↑ or ↓↓ = increased or decreased; ↑↑↑ or ↓↓↓ = highly increased or decreased.

6 Discussion

6.1 General responses to immobilization, glucocorticoids and exercise

Both immobilization and dexamethasone treatment caused loss of muscle weight reflecting muscle atrophy. During immobilization, type I fibers of hind-limb muscles are known to atrophy more than type II fibers (Edgerton *et al.* 1975), whereas corticosteroid treatment causes morphological and functional changes especially in fast muscles (Seene & Viru 1982, Seene *et al.* 1988, Nava *et al.* 1996). From the rat hind-limb muscles, SOL is mainly composed of type I fibers, whereas GM, EDL and TA contain mostly type II fibers (Armstrong & Phelps 1984, Delp & Duan 1996). The considerable muscle atrophy that occurred in SOL during immobilization (34 % after 7 days) and in TA during 10-day dexamethasone treatment (15 %) in rats is in accordance with these earlier findings, although the degree of atrophy was only slightly more pronounced in fast EDL than in slow SOL during dexamethasone treatment (11.5 % and 8.5 % decrease in muscle weights after 10 days, respectively). The observed atrophy in both SOL and GM during immobilization is also in consent with the earlier findings that atrophy is most marked in plantarflexors (Booth 1977, Savolainen *et al.* 1987, 1988). Systemic glucocorticoid treatment causes atrophy of many tissues and reduces the food and fluid intake of animals (Nava *et al.* 1996), thereby causing rapid loss of body weight. Weight loss was observed also in the present study as the body weights of all dexamethasone-treated animals decreased during the whole experimental period. During immobilization the body weights were slightly decreased after one day, possibly caused by the effects of anesthesia on the food and fluid intake of the animals. Normal weight gain was achieved at the 7^{th} day of immobilization. Exercise increased body weight during 10 days, although it did not have any effect on the weight of the leg muscles. In the present studies, the total muscular RNA content decreased during immobilization and dexamethasone treatment. Since the main part of total RNA is of ribosomal type, these findings suggest a rapid inhibition of the translational capacity in muscles during these conditions.

6.2 Synthesis of fibrillar collagens

Collagen synthesis involves numerous stages between the transcription of the genes to the crosslinking of the mature collagen molecules (Fig. 3). As markers of collagen synthesis, the mRNA levels of collagens, activity of prolyl 4-hydroxylase, and the amount of hydroxyproline were measured from rat muscles during immobilization. In neuromuscular diseases, mRNA levels were measured and the amounts of collagen propeptides were analysed semiquantitatively from immunohistochemical stainings made from human muscle biopsies. mRNA levels reflect the gene expression of proteins, although continuous degradation of mRNAs occurs. The data gathered hitherto indicate that the regulation of fibrillar collagen synthesis in cultured cells or in developing organisms occurs through the regulation of mRNA levels rather than through translational control (Bennett & Adams 1990). However, sufficient activity of prolyl 4-hydroxylase seems to be essential for collagen synthesis, since inhibition of this enzyme leads to synthesis of non-functional underhydroxylated collagen degraded extracellularly (Oikarinen *et al.* 1986). A few other enzymes including hydroxylysyl galactosyltransferase, galactosylhydroxylysyl glucosyltransferase and lysyl oxidase also participate in the posttranslational modifications of collagens, but they were however not measured in this study. Collagen propeptides reflect the synthesis of collagen types I and III, since these collagens are synthesized as procollagens, and the propeptides from each end of the molecules are removed by specific proteinases after their secretion from the cell (Prockop *et al.* 1998). A common assumption in studies of collagen turnover is that measured hydroxyproline derives from collagen. Although it is known that some other proteins contain hydroxyproline, their contribution to the total hydroxyproline content of a tissue is probably negligible (for bibliography, see Mays *et al.* 1991). Thus, the decreased collagen mRNA levels with decreased activities of prolyl 4-hydroxylase during immobilization suggest a marked decrease in the synthesis of type I and III collagens in rat plantarflexors, with a slight or insignificant decrease in dorsiflexors. The increased mRNA levels for type I and III collagens with increased staining intensities for PINP and PIIINP in humans suggest increased synthesis of these fibrillar collagens in both polyneuropathy and polymyositis muscles, although the analyses of PINP and PIIINP were only semiquantitative. However, the increased staining intensities were so distinct that elevation in the amount of propeptides in these diseases seems probable.

6.3 Expression of MMPs

Controlled breakdown of ECM is essential for development, tissue repair and angiogenesis (Kähäri & Saarialho-Kere 1999). Excessive breakdown apparently has a pathogenetic role in degenerative diseases, e.g. arthritis and periodontitis. Gene mutations causing inherited human disease were recently found in the MMP-2 gene. These mutations cause functional MMP-2 to be abolished resulting in nodulosis, arthropathy and osteolysis (NAO) syndrome. (Sternlicht & Werb 2001) In skeletal muscle, MMPs have a role in the degeneration-regeneration process after muscle injury. MMP-9

expression seems to be related to the inflammatory response and activation of satellite cells, whereas MMP-2 has a role in the regeneration process (Kherif *et al.* 1999). Present studies on the degradation of type IV collagen during immobilization and glucocorticoid treatment were limited to MMP-2 and MMP-9. From the MMP family, MMP-1, MMP-2 and MMP-16 are continuously expressed in normal skeletal muscle (Singh *et al.* 2000, Balcerzak *et al.* 2001). The fibroblasts in skeletal muscle may also express tiny amounts of MMP-13 and MMP-15 (Balcerzak *et al.* 2001), whereas expression of MMP-3, MMP-7 and MMP-14 has not been observed (Singh *et al.* 2000, Balcerzak *et al.* 2001). The other MMPs have not been studied in normal skeletal muscles. Of these MMPs that are expressed in skeletal muscle, both MMP-13 and MMP-16 degrade type III collagen, MMP-13 being also able to cleave type I collagen (Table 1). MMP-15 activates proMMP-2, but has no direct role in the degradation of collagens. The MMP-mediated degradation of type IV collagen in normal healthy skeletal muscle seems to occur mostly through the activity of MMP-2, since none of these other MMPs found in normal muscle are able to degrade type IV collagen. In addition to the MMPs continuously expressed in skeletal muscle, expression of MMP-3, MMP-7, MMP-9, MMP-10 and MMP-11 has been reported in human skeletal muscles with neuromuscular diseases (Schoser & Blottner 1999, Kieseier *et al.* 2001). Of these MMPs, MMP-3, MMP-7, MMP-9 and MMP-10 are able to degrade type IV collagen, and therefore their role in the degradation of basement membranes in disease conditions may be more significant than the role of continuously expressed MMP-2.

While the increased gene expression of MMP-2 caused an elevated quantity of proMMP-2 and MMP-2 during immobilization in rats, the increased gene expression did not result in elevated quantity during polyneuropathy in humans. Since MMP-2 is the only MMP known to degrade type IV collagen during immobilization, a rapid increase in its expression and synthesis is probably essential for the basement membrane remodeling. The degrading activity of other MMPs expressed during muscle diseases, e.g. the observed elevation in the expression of MMP-9, may overtake the role of MMP-2 in this remodeling, thus making the increase in MMP-2 level unnecessary. The expression of MMP-2 during neuromuscular diseases is contradictory, since no changes to control muscles were found during polymyositis, dermatomyositis, sporanic inclusion myositis, amyotropic lateral sclerosis, spinal muscle atrophy or chronic axonal neuropathies in two studies (Schoser & Blottner 1999, Kieseier *et al.* 2001), whereas Choi & Dalakas (2000) observed increased MMP-2 expression during polymyositis, dermatomyositis and sporanic inclusion myositis. The increase in proMMP-2 was even more pronounced than the increase in proMMP-9 expression during these diseases (Choi & Dalakas 2000). Possible explanations for the differences in these results may be I) the methods used in Choi & Dalakas (2000) were immunocytochemistry and gelatine zymography, whereas Schoser & Blottner (1999) used only immunohistochemistry and did not observe any MMP-2 in normal muscles either, and II) Kieseier *et al.* (2001) used non-inflammatory muscular dystrophy as controls. No previous results are available on the synthesis and expression of MMPs in polyneuropathies. Schoser & Blottner (1999) studied MMP-2, MMP-7 and MMP-9 in late stages of chronic axonal neuropathy with immunohistochemistry and found that the staining for MMP-9 was increased. In the present study, the gene expression and protein level of MMPs were studied in the early stages of polyneuropathy. These findings of increased expression of (pro)MMP-9 both in

the early and late stages of neuropathy possibly suggest a significant role of MMP-9 in the pathogenesis of neuropathies. Dexamethasone treatment decreased the quantity of proMMP-2 in rats, starting with the fast EDL and TA muscles and resulting in a marked decrease in all muscles after 10 days. The decline seems to be caused by other factors than decreased gene expression, since a significant decrease in the mRNA level of MMP-2 started to occur somewhere between three and ten days in TA, and not even at that time in other muscles.

The activity of muscular MMPs was not measured in this study, although it is mentioned in original studies II and III. It is true that the bands observed in gelatin zymography gels are the results of gelatinolytic activity. However, the observed gelatinolytic activity does not relate to the activity *in vivo*, since all latent forms that are inactive in muscle are activated in the gel. The measured density of the proMMP and MMP bands thus reflect the quantity of MMPs in muscle samples, not the activity of MMPs. In the interpretation of zymography results, the term “activity” is widely used in the literature. This misinterpretation has now been corrected in this summary.

6.4 Inhibition of MMPs

Inhibition of the activation and activity of MMPs is very important for the controlled breakdown of ECM. Therapeutic use of TIMPs for the inhibition of the pathogenetic effects of MMPs in tumor growth and angiogenesis is of great clinical interest. However, their use in therapy has so far been disappointing (Whittaker *et al.* 1999, Baker *et al.* 2002), since they may even promote tumor growth (Sternlicht & Werb 2001). The possibility to use inhibitors of MMPs in degenerative diseases such as osteoarthritis has also been under research (Cimpean & Caloianu 1997). The inhibition of MMPs occurs through binding of TIMP to the active part of the enzyme or inhibiting the activation of the latent form by binding to the proMMP (Birkedal-Hansen 1995). All four TIMPs are known to inhibit all MMPs with the exception of TIMP-1, which inhibits poorly MT-MMPs and MMP-19. Although TIMP-1, TIMP-2 and TIMP-3 are expressed in skeletal muscle (Singh *et al.* 2000, Balcerzak et al 2001), the present studies concentrate mainly on the changes in TIMP-2. TIMP-2 is known to bind most effectively to MMP-2, while TIMP-1 and TIMP-3 have other major substrates (Gomez *et al.* 1997). TIMP-2 inhibits MMP-2 activation and activity in high concentrations and activates proMMP-2 in a MT-MMP/TIMP-2 complex in low concentrations. Although MT1-MMP is not expressed in this tissue (Balcerzak *et al.* 2001), TIMP-2 is known to form complexes with other MT-MMPs as wel,l thus activating proMMP-2 (Sternlicht & Werb 2001). Of the MT-MMPs, gene expression of MT3-MMP is observed in skeletal muscle (Balcerzak *et al.* 2001), and protein level expression of some of the MT-MMPs can probably be assumed. Because MMP-2 activation is the dominant function of TIMP-2 in low-to-moderate concentrations (Sternlicht & Werb 2001), the decrease in TIMP-2 quantity in plantarflexors during immobilization probably does not have any effect on MMP-2 inhibition. Therefore the observed increase in mRNA level and expression of MMP-2 leads to increased degradation of type IV collagen. For the same reason, it can be postulated that the

decreased expression of TIMP-2 during dexamethasone treatment does not affect the activity of MMP-2.

The expression of TIMP-1 is regulated by various growth factors, cytokines and hormones including glucocorticoids (Kähäri & Saarialho-Kere 1999), and therefore its quantity was measured in rat muscles during glucocorticoid treatment and exercise. However, TIMP-1 quantity was not changed during dexamethasone treatment, whereas the quantity of TIMP-2 was markedly decreased. While exercise caused no change, or even an increase in TIMP-2 quantity, endurance running decreased the quantity of TIMP-1 in EDL.

6.5 Type IV collagen turnover

Although all studied conditions, immobilization, dexamethasone treatment and neuromuscular diseases, cause muscular atrophy and loss of muscle function, the processes in remodeling of the ECM seem to be quite different. Immobilization is the only condition that causes complete disuse, and probably therefore the downregulation of collagen expression was most pronounced in this condition. The pathology of polyneuropathy could be presumed to be somewhat similar to immobilization, because it also causes partial disuse of the muscle due to diminishing neural stimulation. However, on the contrary to immobilization, the gene expression of collagens is increased in polyneuropathy and, despite the enhanced degradation, type IV collagen seems to accumulate in the basement membranes of muscle cells and capillaries of these human muscles. The reason why only type IV collagen was accumulated in the muscles and not fibrillar collagens is not clear. The underlying diseases, e.g. diabetes, may have a role also in the muscular ECM remodeling, since diabetes is known to cause increased type IV collagen expression in nerves and renal microvessels (Bradley *et al.* 2000, Tsilibary 2003). Accumulation of type IV collagen in the basement membranes of muscle cells and capillaries is physiologically disadvantageous, since it complicates the transportation of nutrients and cellular excreta between blood and cells, and complicates the pathology of the disease even further. The regulation of ECM remodeling during dexamethasone treatment differs from the other conditions because glucocorticoids have direct effects on the cytokines and transcription factors regulating collagen synthesis and degradation (Xu *et al.* 2001). The decreased gene expression and degradation of type IV collagen in dexamethasone-treated rats was probably caused by the inhibitory effect of dexamethasone on the overall synthesis of proteins (Rannels *et al.* 1980, Long *et al.* 2001).

6.6 Compensatory effects of stretch and exercise

Immobilization and glucocorticoid treatment cause muscle atrophy and loss of muscle proteins. Muscle stretch during immobilization is known to prevent muscle wasting and

even induce muscle growth (Goldspink 1977, Savolainen *et al.* 1988). In the present study, muscle stretch partly prevented muscle atrophy during immobilization in rat plantarflexors: SOL and GM. In these muscles the overall loss of muscle weight was more rapid than in the dorsiflexors: EDL and TA. Stretch also partly prevented most of the changes in mRNA level, prolyl 4-hydroxylase activity and type IV collagen concentration, and totally prevented the increase in proMMP-2 and MMP-2 quantity in plantarflexors. Endurance running completely prevented the loss of muscle weight during dexamethasone treatment in rat SOL, and partially in EDL. This is in accordance with earlier studies on endurance running during glucocorticoid treatment (Hickson & Davis 1981, Seene & Viru 1982, Falduto *et al.* 1992). Endurance running might be an effective stimulus especially for the slow SOL, whereas interval-type uphill running seems to lack all effects on any of the muscles. Endurance running also partly prevented the increase in type IV collagen concentration in one muscle, while uphill running had no preventive effects on collagen turnover. At first, exercise combined with dexamethasone treatment seemed to be too strenuous for the animals, since their body weights decreased even more than with sole steroid treatment. This negative effect of exercise disappeared when treatments continued for ten days. The prevention of the anti-anabolic changes in skeletal muscle during glucocorticoid treatment is clinically very relevant, since glucocorticoid treatments are commonly used in various conditions, e.g. in the therapy of fibrotic conditions of the liver (Tanner & Powell 1979), lung (Turner-Warwick *et al.* 1980) and skin (Griffiths 1966), and e.g. organ transplant therapy (Hickson & Marone 1993). For the functional capacity of these patients, it is important to prevent as much as possible the negative changes in muscle tissue.

The utilization of stretch during immobilization in rats seems to have more preventive effects than light exercise during dexamethasone treatment. The exercise might have been too mild to prevent the changes in ECM during the first ten days, although prevention of muscle atrophy was observed. Longer-lasting exercise might have had more effect on the progressive muscle loss. Even though the exercise protocols were light, they probably correlate well to the exercise that can be used as daily exercise in human patients. With more strenuous exercise the effects might have been more pronounced, although their value would have been purely theoretical. However, exercise itself was able to cause increased mRNA level of type IV collagen in SOL, which is in accordance with earlier findings showing that endurance training increases total collagen biosynthesis (Takala *et al.* 1983), and that even a single bout of strenuous exercise can cause an increase in the mRNA levels of collagens I, III and IV in rat skeletal muscles (Koskinen *et al.* 2001).

The problem with immobilizing muscles in lengthened positions is the shortening of the antagonist muscles. At least in rat legs, it seems to be most advantageous to use full plantarflexion, since musculoskeletal anatomy causes dorsiflexors to be shortened much less in plantarflexion than plantarflexors in dorsiflexion (Witzmann *et al.* 1982). Dorsiflexors seem to be able to tolerate the slight shortening, whereas plantarflexors may even benefit from the stretch. This is definitely practical when the ankle is immobilized, and the same idea could be utilized in the immobilization of other joints as well.

7 Conclusions

This study confirms the previous findings of decreased collagen biosynthesis, and brings new knowledge of type IV collagen turnover during immobilization. While the synthesis of collagens is decreased during immobilization, the degradation of type IV collagen is simultaneously increased, leading to decreased type IV collagen concentration. The substantially increased expression of MMP-2 and its proenzyme form is mediated by increased gene expression of the enzyme itself, possibly together with the increased activation of MMP-2 by diminished TIMP-2 levels. These results suggest strictly regulated net degradation of the major basement membrane component, type IV collagen, during the first week of immobilization. It would be interesting to see the early responses of type IV collagen, MMP-2 and TIMP-2 to remobilization. Future studies are also needed to explore the role of TIMP-2 in the activation and inhibition of MMP-2 in muscle tissue during normal turnover and in diseases.

The effects of glucocorticoids on skeletal muscle collagens have not been studied previously. The gene expression of type IV collagen decreases during 10-day dexamethasone treatment. Diminished gene expression of MMP-2 causes the quantity of proMMP-2 to be decreased, although the inhibitory activity of TIMP-2 towards proMMP-2 and MMP-2 is also decreased. The content of type IV collagen is not changed due to the decrease in both gene expression and degradation of this protein. This deceleration of type IV collagen turnover is probably caused by the dexamethasone-induced inhibition of overall protein synthesis, and the inhibition of transcription factors inducing the synthesis of MMPs. Thus, while they are clinically used as anti-inflammatory agents, glucocortoids do not seem to cause excessive loss of the basement membrane protein, type IV collagen.

The present study confirms the previous results of increased gene expression of collagens in polymyositis and polyneuropathy. The results further suggest type IV collagen accumulation not only in polymyositis but also in polyneuropathy. This accumulation of type IV collagen is physiologically disadvantageous, since thickening of the basement membranes of capillaries and muscle fibers complicates the transportation of nutrients and cellular excreta between blood and muscle cells. The increase in type IV collagen concentration seems to be caused by increased gene expression of type IV collagen with constant degradation by MMP-2. Underlying diseases, e.g. diabetes, in

polyneuropathy patients may have a role in the observed increase in type IV collagen concentration. Further research could be conducted to find out the possible roles of systemic diseases on skeletal muscle connective tissue. Unexpectedly, increased quantity of proMMP-9 with increased staining intensity of MMP-9 in some cells was observed in polyneuropathy muscles. The possible role of MMP-9 in the pathogenesis of polyneuropathy could be an interesting area for future research.

The changes in collagen synthesis and degradation in rats can be partly prevented by immobilizing muscles in lengthened positions. Stretch applied in leg muscles during immobilization has preventive effects especially on the plantarflexors, which are the muscles most sensitive to immobilization. The exercise used in this study did not prevent the anti-anabolic effects of 10-day dexamethasone treatment in rats, even though exercise itself had enhancing effects on type IV collagen gene expression and TIMP-2 quantity. To find out whether exercise has any compensatory effects on the glucocorticoid-induced changes, more strenuous or prolonged exercise could be used in future studies. It would also be interesting to find out the effects of exercise prior to glucocorticoid treatment.

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