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Relaxin and [beta]-estradiol induce collagenase and stromelysin expression in temporomandibular joint disc explants

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Relaxin and β-estradiol Induce Collagenase and Stromelysin Expression in Temporomandibular Joint Disc Explants

by

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THESIS

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Relaxin and β-estradiol Induce Collagenase and Stromelysin Expression in Temporomandibular Joint Disc Explants.

Trang T. Duong, D.D.S., M.S.

Abstract

Because of the age distribution of temporomandibular disorders, a role of female reproductive hormones in their etiology has been postulated. However, little evidence in support of this hypothesis has been presented and the mechanisms of action of these hormones on joint tissues have only been partially characterized. We have previously demonstrated that relaxin, a 6 kDa polypeptide hormone induces the matrix metalloproteinases (MMPs) collagenase-1 and stromelysin-1 in isolated rabbit temporomandibular joint (TMJ) disc cells, and that this response is potentiated by β-estradiol priming of the cells (Kapila and Xie, Lab Invest, 78:925-938, 1998). In this study we determined whether rabbit TMJ disc cells in their native matrix environment show a MMP-inductive response to relaxin and β-estradiol similar to that observed previously in isolated disc cells, and quantitated proteoglycan concentration in disc explants in response to these hormones. TMJ discs were retrieved from 20-week old female New Zealand white rabbits and cultured in serum-free medium (αMEM+0.2% LAH) alone or in medium containing 0.1 ng/ml of relaxin or 20 ng/ml of β-estradiol or relaxin plus β-estradiol. The disc conditioned media was collected after 48 hours and analyzed by substrate zymograms and Western immunoblots for MMPs. In agreement with our previous findings on isolated disc cells, relaxin produced a 1.5-fold increase in collagenase expression and approximately a 1.9-fold induction in stromelysin expression in disc explants. However, in contrast to our findings on isolated disc cells which showed a slight decrease in MMPs by β-estradiol, this hormone induced collagenase and stromelysin expression in disc explants. Additionally, our findings revealed a lack of potentiation by β-estradiol of relaxin's induction of MMPs which was observed in the previous study. We also demonstrated that

relaxin and β-estradiol plus relaxin-mediated increases in these MMPs were paralleled by a loss in disc GAGs. However, induction by β-estradiol of collagenase-1 and stromelysin-1 was not accompanied by changes in GAG staining within the disc, which may be attributed to increase GAG/proteoglycan synthesis. Although our ^{35}S studies were not significant, the β-estradiol group demonstrated a trend toward increased GAG synthesis compared to the baseline control, relaxin, and β-estradiol plus relaxin. These studies show that the female reproductive hormones relaxin and β-estradiol induce specific MMPs in disc explants, and that this response differs partially from that of isolated cells. Our findings implicate relaxin and β-estradiol in the degradative remodeling of the TMJ disc. Supported by NIH-NIDR R29 DE10993.

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I. BACKGROUND, SIGNIFICANCE AND SPECIFIC AIMS

Introduction

Temporomandibular disorders (TMDs) are a spectrum of diseases involving the temporomandibular joint (TMJ), muscles of mastication, and associated structures (McNeill 1993). Clinical signs of TMJ diseases include joint clicking, popping, locking of the jaw, pain in the muscles of mastication and/or TMJ, and difficulty in chewing. Despite these often severe manifestations of TMD, the etiology and pathogenesis of these diseases remain unknown. Furthermore, the variable clinical findings, the cyclic nature of the symptoms, the aggravation of the symptoms during and after dental treatment, and the inconsistent skeletal and dental relationships make these patients difficult to manage. Therefore, there exists a need to develop methods that recognize these diseases early and that provide an objective basis for diagnosis of TMJ-related diseases. This objective can be realized through the identification of specific agents that cause or predispose to TMJ diseases. Whereas the determination of causative factors remain elusive, some concepts on the potential etiologic and predisposing factors for TMJ diseases can be developed by examining the epidemiology of these diseases.

The gender and age bias of TMDs may indicate potential etiologies

Despite the unknown pathophysiology of TMDs, these diseases have a significant female to male predilection. In population studies TMDs are known to have a female to male ratio of 2:1 to 6:1 (Solberg et al. 1979; Solberg 1982; McNeill 1993), while in clinical studies this ratio is even further skewed to as high as 8:1 to 10:1. Furthermore, in contrast to diseases of other joints which also have a higher predilection for females but occur primarily in the postmenopausal years, a large proportion of women with TMDs are between 16 and 45 years of age (Von Korff et al. 1988; Carlsson and LeResche 1995).

Although the reasons for this sexual dimorphism and age distribution remain unclear, several theories have been proposed. One hypothesis suggests that women have a higher reporting frequency and tend to seek treatment more readily than men (Dworkin 1983; DeLeeuw 1993; Lipton et al. 1993). This has been disputed by studies which have shown that women seek medical care less often or at equal levels as males (Davis 1981; Von Korff et al. 1991). Further evidence that female predilection for these diseases in not related to the treatment-seeking behavior of women is that women have more problems related to joints and musculoskeletal structures in general, and that the incidence of internal TMJ derangement is indeed higher in women than in men (Oberg et al. 1971; Solberg et al. 1985).

A second hypothesis states that women are more likely to develop TMJ disease due to a higher incidence of systemic joint laxity and the accompanying hypermobility of joints (Buckingham et al. 1991; Westling 1992). Joints that are unduly lax may be injured by minor trauma which would cause little harm to joints of normal stability (Kirk et al. 1967; Bird et al. 1978). Indeed, the prevalence of hypermobility in one or more joints is markedly higher in females than in males, which may explain the higher clinical incidence of both osteoarthritis and internal derangement of the TMJ in women than in men (Westling 1992; Larsson et al. 1993). The ligaments of hypermobile joints have lower quantities of type I collagen than normal joints, suggesting a possible structural and molecular basis for this phenomenon (Child et al. 1984; Westling 1992).

Finally, some investigators (Aufdemorte et al. 1986; Milam et al. 1987) have suggested the potential role of female reproductive hormones, particularly estrogen in the etiology of TMJ diseases. Emerging epidemiologic evidence suggests the role of exogenous hormones, such as those in oral contraceptives in TMDs (Aufdemorte et al. 1986; Milam et al. 1987; LeResche et al. 1993; LeResche et al. 1997). Epidemiologic studies (Spector et al. 1988; Spector and Campion 1989; Hannan et al. 1990; Spector et al. 1991) on other joints also show an increased rate of hysterectomies and gynecological

operations and estrogen supplementation therapy in patients with osteoarthritis than in controls, providing evidence for a potential link between increased estrogen levels and osteoarthritis. The role of estrogen in TMDs is also implicated by findings showing that the use of exogenous hormones, such as those in estrogen replacement therapy is associated with TMD pain in women (LeResche et al. 1997), and by the decreased prevalence of these disorders in postmenopausal women (Carlsson and LeResche 1995). However, no direct evidence exists linking female reproductive hormones to TMJ disease and little information is currently available of the mechanisms by which these hormones may cause joint diseases. Additionally, the potential role of other less known female reproductive hormones in TMJ disease have not been examined. Of particular interest in this regard is relaxin, which is found systemically in cycling and pregnant women but not in men, which modulates matrix remodeling in uterine and cervical tissues, and is associated with pelvic joint hypermobility in women during pregnancy and parturition (MacLennan 1991). The activities of this hormone on connective tissues and its high systemic levels in cycling and pregnant women suggest its potential role in TMJ disease.

Is there a relationship between female reproductive hormones, joint hypermobility and TMDs?

Although no study has been done to examine the association between systemic relaxin levels and generalized joint hypermobility, several studies have suggested a strong relationship between systemic levels of relaxin during pregnancy and postpartum pelvic joint hypermobility and pain (MacLennan 1991; Saugstad 1991). These findings indicate a likely association between high serum levels of relaxin and pelvic pain and joint laxity. This conclusion is supported by *in vivo* findings demonstrating that vaginal application of synthetic relaxin in rats and pigs causes an elongation and a decrease in density and integrity of collagen bundles in the interpubic ligament (Sherwood et al. 1993).

Further evidence for a potential association of relaxin and β-estradiol with generalized joint hypermobility and TMJ diseases is provided by our clinical studies on women with or without symptoms of TMJ disorders (Peikoff 1995). These studies show that symptomatic women with the severest manifestations of TMJ disease, including pain and osteoarthritis, have the highest serum levels of relaxin and β-estradiol as compared to women with less severe manifestations of the disease. Furthermore, as compared to asymptomatic subjects, women with TMJ symptoms had significantly greater ($P < 0.05$) systemic joint hypermobility, which has previously been shown to be closely associated with serum levels of relaxin (Calguneri et al. 1982; MacLennan 1991). Moreover, an association between pregnancies and TMJ disease was noted with both the proportion of women reporting previous pregnancies (44% vs 10.5%) and the mean number of pregnancies (0.8 vs 0.1) being significantly greater ($P < 0.05$) in symptomatic than in asymptomatic women. While these studies provide some evidence for a potential association of specific female reproductive hormones, systemic joint laxity and TMJ disease, direct evidence of the involvement of these hormones in TMJ diseases is still lacking. However, our recent findings (Kapila and Xie 1998) Huh et al., unpublished data) on the effects of relaxin and β-estradiol on joint ligaments and cells suggest a mechanism by which these hormones may contribute to joint hypermobility or TMJ degeneration.

Relaxin is a potent mediator of matrix remodeling activity

Human relaxin exists as two gene products, H1 and H2. Relaxin H1 functions to relax smooth muscles (Bryant-Greenwood and Schwabe 1994). In contrast, relaxin H2 effects the composition of connective tissues by modulating the turnover of collagen and proteoglycans. Because of the known effects of relaxin H2 on matrix turnover and its association with joint hypermobility our study focuses on this form of relaxin. Relaxin H2 is a 6kDa polypeptide which is structurally related to the insulin family of hormones, but

with distinct activities from other members of this family (Mercado-Simmen et al. 1982; Olefsky et al. 1982). In humans, relaxin is produced by the corpus luteum, decidua, chorion, and breast (MacLennan 1991; Bryant-Greenwood and Schwabe 1994). Systemic levels of relaxin are high in females during and following pregnancy, but it has not been detected systemically in men. The average baseline levels of relaxin in cycling women are 30 pg/ml and peak at 150 pg/ml in the midluteal phase (Stewart et al. 1990). In pregnant women, the serum levels of relaxin peak immediately prior to parturition and often exceed 1000 pg/ml (Eddie et al. 1986; Stewart et al. 1990).

Most of the known activities of relaxin are related to the reproductive system. This hormone mediates uterine and cervical matrix remodeling during the menstrual cycle, pregnancy, and parturition by modulating the synthesis and degradation of their extracellular matrices. Compositional changes of the cervix have been shown to be correlated with increased levels of estrogen and relaxin during pregnancy (Sherwood et al. 1980). Relaxin also alters the characteristics of cervical tissues and interpubic ligament by increasing the water content and reducing the concentrations of glycosaminoglycans (GAGs) and collagen (Cullen and Harkenss 1960; Hall et al. 1990; O'Day et al. 1991). Estrogen-priming potentiates relaxin's matrix remodeling activity of the uterus and cervix by further decreasing their content collagen and GAGs (Zarrow and Yochim 1961; Hall and Anthony 1993). In contrast, pretreatment with progesterone does not modulate relaxin-induced changes in cervical composition and distensibility (Cullen and Harkenss 1960; Hall and Anthony 1993). The basis for this increased responsiveness of estrogen-primed tissues to relaxin is not fully understood; however it is likely that this occurs partly through the upregulation of relaxin receptors (Mercado-Simmen et al. 1982; Mercado-Simmen et al. 1990).

Although the effects of relaxin on the histochemical composition and collagen expression in uterine and cervical tissues has been well defined (Bryant-Greenwood and Schwabe 1994), the role of proteinases in hormone-mediated degradation of these tissues

has only been partially characterized (Toole 1991; Kapila and Xie 1998). Additional insights into the role of relaxin-induced MMPs in tissue turnover are provided by studies on cells from non-reproductive tissues such as the skin (Unemori and Amento 1990), and more recently those from specific joint tissues (Kapila and Xie 1998) (Huh et al., unpublished data). Thus, in dermal fibroblasts, relaxin decreases the synthesis of type I collagen and tissue inhibitor of metalloproteinases-1 (TIMP-1), while concomitantly increasing the expression of the matrix metalloproteinase (MMP) collagenase (Unemori and Amento 1990).

Matrix metalloproteinases as mediators of tissue degradation

Mammalian proteinases are divided into four main classes according to their enzymatic action on the various components of the extracellular matrix. Although each of the four classes of proteinases, namely serine, cysteine, aspartic, and metallo found in mammalian cells are involved in matrix destruction, the majority of the degradation is mediated by MMPs (Vincenti et al. 1994). These enzymes comprise a multigene family of at least 21 members and are so named because they have zinc at their active sites and require calcium for activity (Vincenti et al. 1994). They are active at neutral pH and are synthesized and secreted in a latent proenzyme form. Their activation involves proteolytic cleavage of a propeptide domain at the N-terminus of the molecule. MMPs can be grouped into 4 main classes. Group 1 are the collagenases that include interstitial collagenase (MMP-1) and neutrophil collagenase (MMP-8), whose major substrates are the fibrillar collagens types I, II, and III. Group 2 contains the gelatinase or type IV collagenases. These are the 72-kDa gelatinase A (MMP-2) and the 92-kDa gelatinase B (MMP-9), which degrade gelatin and type IV collagen in basement membranes. The third group is made up of the stromelysins: stromelysin 1 (MMP-3), stromelysin-2 (MMP-10), stromelysin-3 (MMP-11), and PUMP-1 (MMP-7). The stromelysins are active against a broad spectrum of substrates such as proteoglycans, laminin, fibronectin, and some collagens. The fourth

group consists of the membrane type of MMPs: MT-MMP-1, 2, 3, and 4. The membrane type of MMPs degrade substrates such as collagens I, II, III, gelatin, casein, laminin, and fibronectin. The activity of MMPs are highly regulated both at the transcriptional level, by their secretion as inactive proenzymes and by their inhibition by specific tissue inhibitors of metalloproteinases (TIMPs). There are presently at least 4 known TIMPs, namely TIMP-1, TIMP-2, and TIMP-3 which inhibit all MMPs, and TIMP-4 which primarily inhibits MMP-1, 2, 3, 7 and 9.

MMPs mediate destruction of joint tissues during arthropathies

MMPs are known mediators of tissue degradation and have been strongly implicated in articular cartilage degeneration during rheumatoid arthritis and osteoarthritis (Krane et al. 1990; Kapila and Xie 1998). Because of the important functions of articular cartilage, its destruction by MMPs contributes substantially to the pathogenesis of joint diseases. The primary functions of articular cartilage are to provide a surface for joint articulation and to withstand joint loading forces. Both of these functions are dependent upon the integrity of the cartilage matrix, which is primarily composed of polyanionic proteoglycans embedded in a meshwork of collagen fibers. Proteoglycans are made up of a protein core to which are attached repeating disaccharide units of GAGs. Because GAGs have a large number of anionic charges, they readily absorb water, producing an internal swelling pressure which is resisted by the tensile collagen fiber network. The net effect of this phenomenon is to provide a cushion against compressive forces, enabling cartilage to retain its original form when load is released (Christensen and Ziebert 1986). Furthermore, the high viscosity and low compressibility of proteoglycans allow them to serve as an ideal lubricating fluid for joints. In contrast to the compressive loads sustained by proteoglycans, fibrilar collagens provide tensile strength to cartilage. The overall physical properties of cartilage that enable it to act as a hydrated fiber-reinforced composite are determined by the complex interactions between collagen and proteoglycans.

Because of the need for cartilage to adapt to changing loads and functions, its matrix macromolecules are in a constant state of turnover. This remodeling of cartilage matrix macromolecules is mediated by chondrocytes. These cells maintain their matrices in normal cartilage by balancing the rate of synthesis with the rate of degradation. Any change in this equilibrium affects the functional integrity of the cartilage (Cawston 1996). The MMP family of proteinases expressed by chondrocytes appear to contribute substantially to both the normal remodeling of cartilage in healthy joints and to its net degradation during disease.

The destruction of articular cartilage is a pathological event which occurs in all joint diseases and leads to disruption of normal joint function (Karran et al. 1995). Numerous studies have reported that the MMP family of enzymes contributes substantially to joint tissue degeneration during inflammatory joint diseases including rheumatoid arthritis and osteoarthritis (Krane et al. 1990; Kapila and Xie 1998). The altered expression of MMPs and their inhibitors by joint fibroblasts and chondrocytes in these arthropathies has largely been attributed to proinflammatory cytokines, implicating both these cytokines and MMPs in the pathogenesis of joint diseases. However, since these findings relate to ongoing joint disease, they fail to explain how these diseases may be initiated and also do not address the reasons for the unique age and gender distribution of TMJ-related diseases. Our recent studies (Kapila and Xie 1998) that demonstrate for the first time, that relaxin alone or in combination with β-estradiol induces the MMPs collagenase-1 and stromelysin-1 in TMJ disc fibrocartilaginous cells implicate the these hormones in a subset of TMJ diseases in women.

Does relaxin contribute to matrix remodeling in joints?

Synovial joints are one of the potential, and until recently unproved, non-reproductive target sites for the tissue remodeling activities of relaxin. Indirect evidence for the potential effects of relaxin on synovial joints was provided by studies showing

increased systemic joint laxity during pregnancy, which is linked to the concurrent elevation of systemic levels of relaxin (MacLennan et al. 1986). Additionally, studies on the non-synovial pelvic joint or pubic symphysis have offered insights into the potential mechanism for relaxin's modulation of joint tissue turnover. *In vivo* animal studies indicate that the increased mobility of the pubic symphysis arises from relaxin-induced alterations in density, integrity, and organization of collagen fibers and proteoglycan content in the fibrocartilage of the joint (Sherwood et al. 1993). Estrogen plays a critical role in the tissue responses to relaxin, because relaxin mediated relaxation of the pubic symphysis requires estrogen priming in virgin and prepubertal animals (Sherwood 1988). These findings suggest a potential role of relaxin in modulating the turnover of synovial joint matrix macromolecules that could consequently cause alterations in their function. Despite these findings, the role of relaxin in joint disease has remained mostly conjectural and correlative. Furthermore, relaxin's potential modulation of matrix composition and mechanisms of action in contributing to diseases of synovial joints in general and the TMJ in particular has only been recently elucidated.

Relaxin induces MMPs in specific tissues of the TMJ

Because relaxin has been shown to modulate the composition of the pubic symphysis fibrocartilage, synovial joints such as the TMJ that have a large content of fibrocartilaginous tissues, may be particularly susceptible to the matrix remodeling activity of this hormone. On the basis of this hypothesis, we have recently demonstrated that relaxin dose-dependently induces the MMPs collagenase-1 and stromelysin-1 in isolated rabbit TMJ disc cells (Kapila and Xie 1998). Priming of these cells with β-estradiol potentiated their MMP-inductive response to relaxin such that the maximum expression of collagenase-1 and stromelysin-1 occurred at 10-fold lower concentrations of relaxin in estrogen-primed than in unprimed cells. This matrix-degradative response to relaxin is cell-type specific, since this hormone produced a dose-dependent decrease in collagenase-1 and

stromelysin-1 in unprimed and β-estradiol-primed synoviocytes. Furthermore, β-estradiol alone did not alter the expression of these MMPs in fibrocartilaginous cells. These findings may have physiologic relevance since the peak induction of collagenase-1 and stromelysin-1 occurred at concentrations of relaxin found systemically in cycling and pregnant women. Our findings suggest that cells within the TMJ disc may be specific target sites for the matrix-degradative affects of relaxin, an effect which is accentuated by β-estradiol. Because the fibrocartilaginous disc is a structure that is relatively unique to the TMJ, its targeting by relaxin may explain the gender and age distribution of TMJ diseases.

The TMJ disc is biconcave in shape and subdivided into anterior and posterior bands separated by a thinner intermediate zone. The immature disc contains numerous fibroblast-like cells surrounded by a matrix of type I collagen fibers and is referred to as a fibrocollagenous tissue. With age, the fibrocollagenous matrix is gradually replaced by fibrocartilaginous matrix, composed largely of cartilage specific proteoglycans and type I collagen (Kapila 1997). The TMJ disc serves important structural and functional roles such that alterations in its morphology, composition, and spatial arrangements may contribute to aggravation of ongoing TMJ diseases. By targeting its matrix-degradative effects on the TMJ disc, relaxin may cause or contribute to TMJ disease in women of reproductive age. This hypothesis is being tested through a series of studies including the one described in this thesis.

Hypothesis, Specific Aims and Significance

Based on predilection of TMDs for women of reproductive age (Solberg et al. 1979; Solberg 1982; Von Korff et al. 1988; McNeill 1993; Carlsson and LeResche 1995), and the targeted induction of MMPs by relaxin in unprimed and β-estradiol-primed TMJ disc fibrocartilaginous cells (Kapila and Xie 1998), it is possible that specific TMJ disorders in women result from altered systemic levels of relaxin and estrogen. While relaxin's induction of MMPs in disc fibrocartilaginous cells may be important, these

findings do not necessarily indicate that the increased expression of MMPs results in a net loss of disc matrix macromolecules. Additionally, while these results demonstrate relaxin's induction of MMPs in isolated disc cells, it is not yet clear that this response also occurs in cells within their natural matrix environment. Therefore, the purpose of this study was to determine whether relaxin modulates the expression of MMPs and causes a loss of one class of the MMP substrate matrix macromolecules, the proteoglycans, by β-estradiol, relaxin, and β-estradiol plus relaxin in TMJ disc explants. We tested the hypothesis that relaxin's induction of MMPs in disc explants is similar to that of isolated cells in culture and that this is accompanied by a loss in disc matrix macromolecules. The specific aims of this study were to:

1. Determine whether relaxin and β-estradiol mediate changes in expression of MMPs in TMJ disc explants.

2. Evaluate whether relaxin-induced expression of stromelysin is accompanied by a loss of disc GAGs. The levels of disc GAGs were used as a direct measure of changes in proteoglycans.

3. Determine the relative contributions of the relaxin-mediated changes in MMP expression and matrix synthesis to the loss of disc GAGs.

The findings from this study will provide insights into the potential hormonal etiology of TMJ diseases in women and the mechanism by which these hormones may initiate or potentiate TMJ disease. This may, in turn, provide us with a better understanding of the basis for the age and gender distribution of TMDs. The findings of this study could potentially be useful in the development of better diagnostic methods, and biologically rational therapeutic and preventive approaches for TMJ diseases in women.

II. MATERIALS AND METHODS

Materials

Twenty week old female New Zealand white rabbits (*Orcytolagus cuniculus*) were obtained from Nita Bell Laboratories (Hayward, CA) and Grimauld Farms (San Jose, CA) and housed at the Animal Care Facilities at University of California San Francisco. Lactalbumin hydrolysate (LAH), sodium dodecyl sulfate (SDS), α-casein, Tris-base, sodium hydroxide, glycine, β-estradiol 17-valerate, Papain, N-acetylcysteine, disodium ethylenediaminetetracetic acid (EDTA), chondroitin sulfate A sodium from bovine trachea, Safranin-O, fast Green, sodium sulfate, paraformaldehyde, cetylpyridinium chloride were purchased from Sigma (St. Louis, MO). Triton X-100, commassie blue, acetic acid, potassium chloride and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Gelatin (EIA grade) was purchased from Biorad (Hercules, CA). Dulbeccos minimum essential medium (DMEM) was obtained from Gibco BRL (Gaithersburg, MD.). Acrylamide and bis-acrylamide was from Promega (Madison, WI.). OCT was purchased from Miles Scientific (Naperville, IL). 1,9 dimethylmethylene blue (DMB) was purchased from Molecular Probes (Eugene, OR), ^{35}S was obtained from Amersham Pharmaceuticals. PBS (Ca++, Mg++ free), 100x glutamine, penicillin-streptomycin and fungizone were bought from UCSF cell culture facilities. Minimum essential medium (without Earle's salt, L-glutamine, phenol red) was purchased from Gibco BRL (Life Science Technologies, Grand Island, NY). Recombinant human relaxin was kindly provided by Contends Corporation (Palo Alto, California). The MMP inhibitor GM6001 synthetic and its control analogue GM2454 was kindly provided by Dr. Richard Galardy (Glycomed, Alameda California). Alkaline-phosphatase-conjugated goat anti-mouse antibody and alkaline-phosphatase-conjugated goat anti-rabbit antibody were from Pierce (Rockford, IL). Nitrocellulose membrane, nitroblue tetrazolium (NBT), bromochloro-indolyl phosphate (BCIP), were from BioRad (Hercules, CA). Mouse anti-rabbit-collagenase-1 monoclonal antibody was a gift from Dr. Z. Werb (Frisch et al. 1987; Werb et al. 1989). Mouse anti-

human-stromelysin-1 monoclonal antibody (SL188.2), which is known to crossreact with rabbit stromelysin (Tremble et al. 1993), was the gift of Dr. S. Wilhelm (Wilhelm et al. 1992).

Rationale for Using the Rabbit TMJ

In this study the rabbit TMJ disc was used because the morphology and functional physiology of the rabbit TMJ has been well characterized (Weijs and Dantuma 1981; Weijs and Van der Weilen-Drent 1982; Mills et al. 1988; Savalle et al. 1990), and because the rabbit TMJ serves as a suitable analogue to the human TMJ (Scapino 1983). Furthermore, as with humans, the rabbit TMJ disc possesses anterior and posterior band areas rich in PGs, which are separated by a narrow intermediate zone rich in antero-posteriorly directed collagen fibers (Mills et al. 1988). Finally, previous studies (Kapila and Xie 1998) on disc fibrocartilaginous cell responses to relaxin provide the necessary background necessary for the present investigation.

Retrieval of Discs and Culturing Techniques

All procedures on the rabbits were performed according to the accepted protocol for animal welfare and with approval from the University of California San Francisco Committee on Animal Research. The rabbits were anesthetized with 40 mg/kg of ketamine hydrochloride (Ketalar; Parke Davis, Morris Plains, New Jersery) and 3 to 5 mg/kg of xylazine (Rompan; Rugby Laboratories, Inc., Rockville Center, New York). Intact TMJ discs were retrieved bilaterally from the rabbits under sterile conditions and immediately placed in PBS without calcium and magnesium and containing antibiotics (100 U/ml penicillin and 100 mg/ml of streptomycin) and 100 U/ml fungizone. The synovium attached to the discs was removed under a dissecting microscope and the discs were washed three times in PBS. Each disc was bissected longitudinally such that 4 samples were available from each animal for one control and three different hormone treatments.

Each half disc was weighed and placed in wells of a 96 well culture plate. A 150 µl aliquot of serum-free medium (phenol free MEM with 0.2% LAH, 1x Glutamine, 1x non-essential amino acids, 100 U/ml penicillin and 100 mg/ml of streptomycin) with or without hormones was added to each well and the discs cultured at 37 ^{0}C in 5% CO_2 in air. The discs were exposed to medium alone (C) or 20 ng/ml β-estradiol (E) or 0.1 ng/ml relaxin (R), or 20 ng/ml β-estradiol plus 0.1 ng/ml relaxin (E+R). The rationale for the relaxin concentration chosen for these studies was based on the findings that (1) the peak physiologic concentrations of relaxin in cycling women is 0.1 ng/ml (Eddie 1986; Stewart 1990), and (2) estrogen-primed disc cells produce a maximum induction of MMPs at 0.1 ng/ml of relaxin (Kapila and Xie 1998). After 48 hours of incubation, the disc-conditioned medium was collected and stored at -70 ^{0}C until further analysis for MMPs. For experiments in which discs were stained for GAGs, the discs were fixed overnight in 2% PBS-buffered paraformaldehyde prior to further processing and sectioning. For experiments in which disc GAGs were quantitated by DMB assay, the discs were subjected to papain digestion as described below.

Substrate Zymography and Densitometry

Gelatin and casein substrate zymography were used for evaluation and characterization of disc cell secreted proteinases. Conditioned media from nine separate animals (36 disc sections) with a mean weight of .013 mg (SE ± .009) were used for evaluation and characterization of secreted proteinases. Following standardization of the disc-conditioned medium (CM) by tissue weight, 4x sample buffer (0.25M Tris-base, 0.8% SDS, 10% glycerol and .05% bromophenol blue) was added. The samples were electrophoresed at 15 ^{0}C on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) containing 2mg/ml of gelatin or α-casein as described previously (Heussen and Dowdle 1980; Chin et al. 1985). Following electrophoresis, the SDS was removed by washing the gels in 2.5% Triton X-100 for 30 minutes with one change of washing media.

Because initial studies (see description below) showed that optimal time of incubation for gelatin and casein gels was between 42 to 48 hrs and 62 to 66 hours, respectively, the gels were incubated for these periods of time at 37 ^{0}C in incubation buffer (50 mM Tris-HCl buffer, pH 8, 5 mM $CaCl_2$, 0.02% NaN_3). Following incubation, the gels were stained with 5% Commassie blue and destained in 10% acetic acid and 40% methanol until proteinase bands were clearly visible. Images of the gels were video-digitized by a CCD camera (NEC T1-24A, Japan) and image software (Image Version 1.42, NIH, Bethesda, MD). The imaging of the gels was standardized by capturing them at the same focal length and exposure. The levels of 53/58-kD gelatinolytic activity and 51/54-kD caseinolytic enzyme and their lower molecular weight activated forms were quantified by video-densitometry, as described previously (Kapila et al. 1995).

Quantitative assessments from substrate zymograms are complicated because of the nature of enzyme-substrate kinetics which have been controlled for in only a few studies to date (Overall et al. 1989; Fini and Girard 1990; Kapila et al. 1995). In order to overcome these limitations of substrate zymograms, preliminary experiments were performed to determine the optimum time of incubation for the range of activity observed in the samples. Serial dilutions of a sample with high levels of proteinase activity were electrophoresed and gelatin gels incubated for 26, 42 and 60 hours while casein gels were incubated for 60, 96 and 123 hours (data not shown). The findings showed that the optimal incubation time for gelatin zymograms was approximately 42 hours, and that for casein gels was approximately 60 hours. Therefore, all subsequent zymograms were incubated for these periods of time.

Western Immunoblotting for Collagenase-1 and Stromelysin-1

Using samples from relaxin-treated conditioned media, we identified the 53/58 kD gelatinolytic and 51/54 kD caseinolytic proteinases by Western blots as collagenase-1 and stromelysin-1, respectively. Briefly, the disc-conditioned medium was mixed with 4x

sample buffer and electrophoretically resolved on 10% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose membranes, and the membranes blocked, washed, and incubated for 1 hour with mouse anti-rabbit antibody to collagenase-1 (1:500 dilution in tris-buffered saline) (Werb et al. 1989) or sheep anti-human antibody to stromelysin-1 that also recognizes rabbit stromelysin (1:150) (Tremble et al. 1993). After washing, the membranes were incubated with alkaline-phosphatase-conjugated goat anti-mouse or goat anti-sheep antibodies, respectively. After additional washes, the bands were visualized by incubating the blots in 50 μg/ml nitroblue tetrazolium and 25 μg/ml of bromchloro-indoly phosphate.

Histochemical Staining and Quantitation of GAGs

Following fixation, the discs were washed three times in PBS and frozen in OCT embedding compound with 2-methylbutane and dry ice. Each half disc was positioned in the OCT such that the flat cut end was accessible for sectioning. The frozen discs were sectioned with a cryostat (Jung Frigocut 2880 N, Leica Deerfield, IL), and 2 sections placed per slide with a total of 10 sections analyzed. The slides were labeled with a code such that the examiner was blinded to the treatment group to which the sections belonged. The sections were then stored at -70 ^{0}C until further analysis by Safranin-O staining.

Safranin-O is a stochiometric stain of sulfated glycosaminoglycans, the oligosaccharide component of proteoglycans (Kirviranta et al. 1984). The slides were defrosted for 30 minutes, and the sections fixed in methanol for 10 minutes. The sections were then air dried for 15 minutes following which they were stained with 1% Fast Green solution for 2 to 3 minutes, and then placed in 1% acetic acid for 1 minute. The sections from both the control and treatment groups were then stained with 2% Safranin-O for 2 minutes following which they were dehydrated through successive ethanol washes starting from 70% ethanol up to 200% ethanol. The sections were briefly placed in xylene following which they were mounted with then cover slips.

The stained discs from seven animals (28 disc sections) were analyzed by capturing the images onto Adobe Photoshop 4.0 (Adobe, San Jose CA), from a negative transparency at 1200 dpi using a Microteck ScanMaker III flatbed scanner (Redondo Beach, CA 1996). Following capturing, the autolevels, brightness and contrast were modified in order to enhance the clarity of the image and the areas with GAGs that were stained orange / red (Fig. 1A). The sections were then analyzed by deriving a ratio of the area stained for GAGs to the total area of the disc. This analysis was performed at a magnification of 175%. First a small representative part of the area stained orange / red was selected using the lasso tool with a tolerance of 10 dpi's. The program was then instructed to select all areas stained within this selected range of color and intensity using the "similar" tool, filled with black, and then cut and pasted onto an image analysis program (NIH Image 1.61) (Fig. 1C). The total area of the disc was also selected by using the lasso tool to select representatively stained areas of the disc with a tolerance of 40 dpi's' following which all areas with this color and intensity of staining were selected by the computer using the "similar" tool (Fig. 1B). The entire disc outlined by this procedure was then filled with black and then cut and pasted into the image analysis program. The images were saved in TIFF format and analyzed in NIH image 1.61 using threshold and the binary modes on a scale of 1 pixel per pixel. The total area of the disc as well as area of GAG staining in pixels were determined and used to derive the percentage of positive GAG staining to total disc area by multiplying the ratio of disc staining positive for GAGs to total disc area. This technique was found to minimize the subjectivity involved in manually selecting the area of GAG staining by standardizing the intensity of the Safranin-O staining selected to measure this area. Furthermore, this approach minimized the error involved in manually tracing the entire disc or manually tracing the outline of GAG staining.

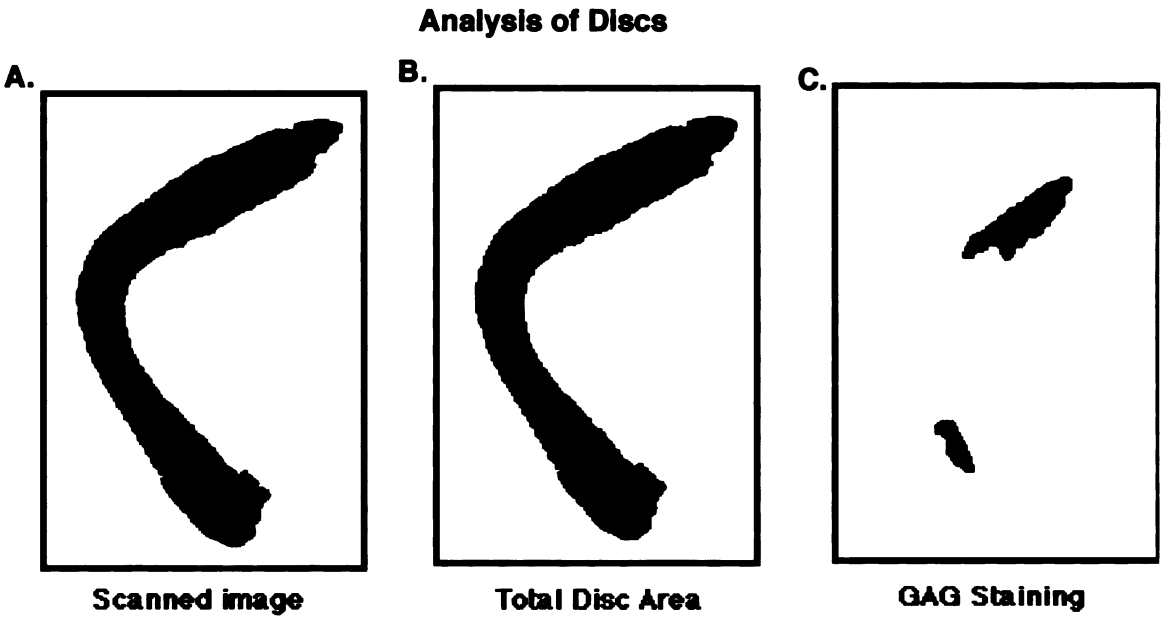

Figure 1: Quantitation of proportion of disc staining for GAGs. Parasagittal histochemically stained section of discs were scanned into Photoshop at a magnification of 1x using a flatbed scanner, following which autolevels were adjusted to enhance the image that was visualized at 100% magnification pasted at 300 x 300 pixels (A). Figure (B) represents the total area of the disc selected by using the lasso tool (tolerance of 40 dpi's). The "similar" tool was used to select the entire disc, and then the image filled with black and copied and pasted onto an image size that was 300 x 300 pixels. Figure (C) represents the areas stained positively for GAGs with Safranin-O. The lasso tool (tolerance of 10 dpi's) was used to select the areas stained red. Once again, the "similar" tool was used to select all areas stained positvely for GAGs. These areas were then filled with black and then copied and pasted onto an image size that was 300 x 300 pixels.

Quantitative Determination of GAGs

The DMB assay was used to quantitate the GAGs retained within the disc following hormone treatment of disc explants. DMB is a strongly metachromatic dye which was initially used for histochemical detection of sulfated GAGs (Taylor and Jeffree 1969). This assay has subsequently been modified to measure the sulfated GAG content in tissue and fluids (Farndale et al. 1986). The ability of sulfated GAGs to produce a metachromatic shift upon binding to the cationic DMB dye is the basis behind this assay (Melrose and Ghosh 1988).

The 1,9-DMB reagent was prepared as described previously (Chandrasekhar et al. 1987). Briefly, 8 mg 1,9-DMB was dissolved in 2.5 ml ethanol followed by the addition

of 1.0 ml formic acid and 1.0 g sodium formate and made up to 500 ml with distilled water. Chondroitin sulfate A sodium from bovine trachea was dissolved at 1 mg/ml in 0.05 M sodium acetate, pH 6.8 and serial dilutions were used to construct a standard curve. Fifty µl of conditioned media from each of the treatment groups were mixed with 200 µl of DMB reagent in 96 well plates and the absorbance was determined with a optical multiwell plate reader (Vmax Kinetic Microplate Reader, Molecular Devices Corporation, Menlo Park CA) at a wavelength of 650 nm. The optical density of the samples was used to determine the proteoglycan content in µg/ml against the standard curve, and the concentration of proteoglycan in conditioned medium was derived by standardizing the proteoglycan content by the total tissue weight in µg/ml/mg.

Determination of Optimal Time for Proteoglycan Digestion by Papain

Since the next series of studies required the dissolution of disc proteoglycans, we performed a study with papain and the DMB-binding assay in order to determine the optimal time for complete digestion of the proteoglycans. The discs from one animal were dissected out as described previously and each disc was bisected longitudinally. The four half discs were then weighed and cultured separately in phenol-free, serum-free medium for 48 hours. They were then placed in 500 µl of 20 U/ml papain solution (10-20 U/mg) in 50 mM sodium phosphate buffer, pH 6.5, containing 2 mM disodium EDTA (Karran et al. 1995) and incubated at 65 ^{0}C. At 2, 4, 6, 8, 10, 17, 26 and 43 hours, 50 µl of the digest were aliquoted out and 50 µl of fresh papain replaced. The DMB-binding assay was done as described above to measure the concentration of proteoglycan, which was standardized by total disc weight. The results showed that after 12 hours of digestion the concentration of proteoglycan in the digest plateaued off (Fig. 2), indicating that the optimal time for complete digestion was at 12 and 24 hours of digestion. Therefore, all subsequent papain digestions were performed at 12 and 24 hours.

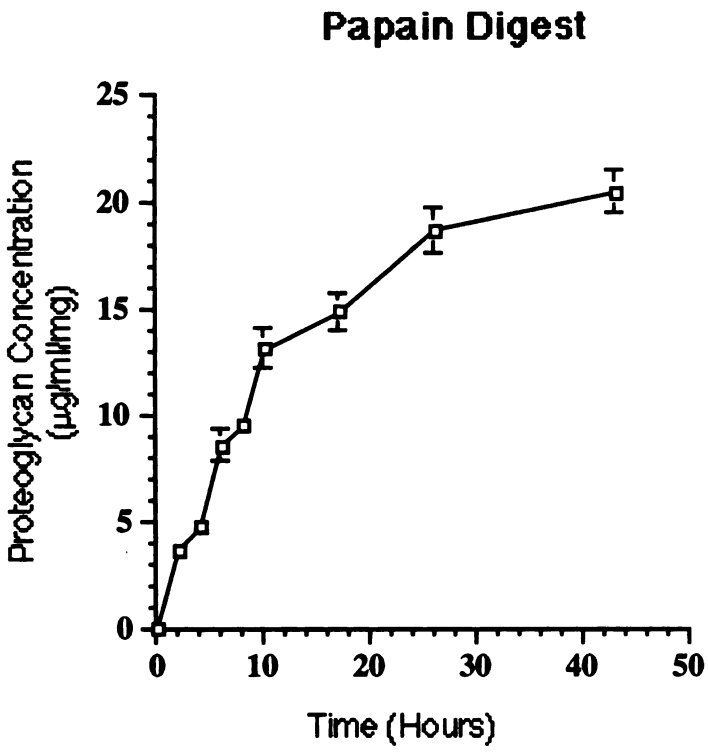

Figure 2: Determination of optimal time for digestion of disc proteoglycans. Levels of proteoglycan in the papain digest taken at 2, 4, 6, 8, 10, 17, 26 and 43 hours were measured for four half discs. Following each time point, 50 µl of the digest was aliquoted out, and 50 µl of fresh papain replaced. The DMB-binding assay was performed on the 50 µl of the digest, and its concentration of proteoglycan was derived from a standard curve. The proteoglycan concentration was standardized by disc weight and the mean (±S.E.) for the 4 disc sections is represented on the Y-axis in µg/ml/mg.

^{35}S Radiolabeling for GAG Synthesis

Because the structure of GAGs consist of numerous repeating sugar units which contain sulfate groups, ^{35}S serves as a good marker for GAG synthesis. Following dissection and washing, the discs from nine separate animals (36 disc sections) with a mean weight of .014 mg (SE ± .001) were incubated at 37 ^{0}C for 6 hrs in 1 ml of phenol free, serum free medium with or without hormones and 165 Kbq (0.0044 mCi) of ^{35}S as described previously (Karran et al. 1995). The discs were then washed three times with phenol free medium containing 1 mg/ml sodium sulphate. The discs were then digested with papain. Papain is a thiol protease from *Carica papaya*. Papain is thermostable and will cleave a variety of peptide bonds. A preliminary study revealed that the time required

for releasing almost all GAGs from the discs was between 12 to 24 hrs (Fig. 2). Additionally, a preliminary study (data not shown) revealed that the levels of ^{35}S were not significantly different at 12 and 24 hours of digestion. Therefore, all subsequent digestions were done at 24 hours. The discs from the control and treatment groups were therefore digested for 12 hrs at 65 ^{0}C in 1 ml of 20 U/ml papain solution (10-20 U/mg) in 50 mM sodium phosphate buffer, pH 6.5, containing 2 mM disodium EDTA (Karran et al. 1995). Following digestion, 100 µl of 5% cetyl pyridinium chloride in 0.3 M potassium chloride solution was added to 500 µl of papain digest and the mixture allowed to stand for 30 minutes at room temperature to precipitate the GAGs. After centrifugation (3000g for 20 minutes), the supernatant was removed and the precipitate dissolved in 600 µl of concentrated formic acid by heating to 70 ^{0}C for 10 minutes. Twenty µl aliquots of this solution were added to 3 ml of scintillation fluid and subjected to liquid scintillation counting. The radioactivity measured in counts per minute was standardized to the total original disc weight as a measure of GAG biosynthesis.

Utilization of MMP Inhibitors to Determine the Contribution of Hormone-induced MMPs to Disc Matrix Degradation

To determine the contribution of MMPs to the loss of GAGs, we performed studies using a synthetic inhibitor. There are two principal ways to decrease the levels of MMPs, namely the inhibition of enzyme activity or inhibition of enzyme synthesis. Since we were studying if relaxin-induced MMPs contribute to disc matrix loss, we utilized the former method using an MMP specific inhibitor. There are several naturally occurring inhibitors called tissue inhibitors of metalloproteinases (TIMPs) that are specific to MMPs. However, the utility of these inhibitors to blocking matrix degradation in tissue explant studies may be limited by their relatively large size and potential inaccessibility to sites within the tissue. Additionally, several chemotherapeutic agents, antibiotics, and synthetic peptides are available that also inhibit the activity of MMPs. The largest group of synthetic

peptide inhibitors are the collagen substrate analogs (Henderson and Davies 1991). All are less than 6 amino acids long, and up to three of the amino acid residues are found on either side of the collagen scissile bond, and the peptide is linked to a Zn^{++} binding moiety. The hydroxamate-based compounds are generally considered to be the most potent inhibitors. The carboxyalkyl and hydroxamate derivatives have a significantly lower IC_{50} for stromelysin than for collagenase (Henderson and Davies 1991). For this study GM6001, a hydroxamic acid dipeptide analogue for MMPs was used. Peptide hydroxamic acids are excellent inhibitors of zinc metalloproteinases (Powers and Harper 1986). The peptide GM 6001 is the hydroxamic acid HONHCOCH2CH(i-Bu)CO-L-Trp-NHMe isomer which has been shown to inhibit skin fibroblast collagenase with K_i of 0.4 nM (Grobelny et al. 1992). Good binding to the enzyme is due to chelation of the active site zinc atom as shown by X-ray crystallography of thermolysin-hydroxamate (Holms and Mathews 1981).

For inhibitor experiments, stock solutions of 400 mM metalloproteinase synthetic inhibitor (MPI; GM6001, 3-(N-hydroxycarbamoyl-2(R)-isobutyl propionyl-L-tryptophan methylamide) and 40 mM control analogue, metalloproteinase inactive control compound (MIC; N-tert-butyloxycarbondyl)-L-leucine-L-tryptophan methylamide) were prepared in DMSO (Leppert et al. 1995; Chin and Werb 1997). The stock solutions of the MPI and MIC were diluted with phenol free media to a working concentration of 100 µM. Discs were incubated in the respective treatment groups along with either MPI (3 separate animals) or MIC (3 separate animals) with a mean weight of .013 mg (SE ±.006). Following 48 hours of incubation, the media were collected and stored at -70 ^{0}C for further analysis. The discs were then washed three times in PBS (calcium and magnesium free) and then weighed. The discs were digested in papain for 24 hours at 65 ^{0}C as described above. The digest was centrifuged at 14,000 rpm (15,000 g) for 15 minutes to obtain a clear solution for the DMB proteoglycan assay. Twenty five µl of the digest from each of the treatment groups was mixed with 200 µl of DMB reagent plus 25 µl of 0.05 M sodium acetate (pH 6.8) in 96 well plates and the absorbance was determined at a wavelength of

650 nm in an optical multiwell plate reader as described previously. The proteoglycan content was determined from a standard curve and its concentration derived by standardizing to total disc weight.

Statistical Analysis

Since substantial differences were noted in results between discs from different animals, all results for MMP levels, GAG staining, proteoglycan concentration and GAG synthesis were standardized within each animal to the respective value from the control disc section. This determination provided data on the fold induction or change in various parameters produced by the three hormone treatments relative to that in the control group. The mean (± S.E.) fold-induction or change of these parameters relative to control baseline levels were plotted and represented as a histograms. The statistical significance for the fold-induction of the measured variables for discs exposed to β-estradiol, relaxin, and β-estradiol plus relaxin relative to control baseline levels was determined by single-factorial analysis of variance (ANOVA), and the intergroup differences were determined by Fisher's multiple comparisons test with the level of significance set at $P < 0.05$.

III. RESULTS

Relaxin and β-estradiol Induce Gelatinolytic and Caseinolytic MMPs in TMJ Disc Explants

Analysis of TMJ disc-conditioned media by gelatin substrate zymograms demonstrated that explanted discs synthesize 4 gelatinolytic proteinases at 92-kDa, 72-kDa, 53/58-kDa and 43-kDa (Fig. 3A, lane 1). On the basis of their inhibition by the metalloproteinase inhibitor, 1-10 phenanthroline these proteinases have previously been characterized as MMPs (Kapila et al. 1995), likely 92 -kDa gelatinase, 72-kDa gelatinase, procollagenase and collagenase, respectively. Indeed, the latter two proteinases have been identified as pro- and active collagenase-1 (MMP-1) in our previous studies (Kapila et al. 1995; Kapila and Xie 1998). The 53/58-kDa gelatinolytic proteinase was confirmed by Western blots to be procollagenase-1 in the present study as well (Fig. 3A, lane 5).

Exposure of the discs to E, R, and E+R did not affect the expression of 72-kDa gelatinases. Although not quantitated, these hormone treatments caused a slight increase in levels of 92-kDa gelatinase expression. Additionally, all three hormonal treatments increased the levels of both the 53/58-kDa (procollagenase-1) and 43-kDa (collagenase-1) gelatinolytic enzymes. This finding was confirmed by video-densitometry of gelatin zymograms from 9 animals (36 disc sections), which showed a greater than 1.5-fold induction of the 53/58-kDa proteinase by E, R, and E+R over control baseline levels (Fig. 3B). The induction of this proteinase by E or R relative to control baseline levels was statistically significant at the $P < 0.05$ level, while that by E+R was significantly higher at $P < 0.01$ level. Although the figure shown (Fig. 3A, lane 4) is not representative of the induction of collagenase by E+R, the mean fold induction from nine separate animals (Fig. 3B) did show on average significantly increased induction by E+R.

Explanted TMJ discs also synthesized 2 caseinolytic proteinases at 92-kDa and 51/54-kDa (Fig. 4A, lane 1). Western immunoblot of a selected sample of disc-

conditioned medium identified the 51/54-kDa proteinase as prostromelysin-1 (proMMP-3) (Fig 2A lane 5) confirming previous findings (Kapila et al. 1995). Densitometric measurements of caseinolytic zymograms from 9 animals (36 disc sections) revealed that the levels of the 51/54-kDa MMP were increased in β-estradiol, relaxin and β-estradiol plus relaxin treated discs as compared to that in untreated control discs. All hormone treatment groups showed an approximately 1.9-fold induction of the 51/54-kDa proteinase relative to control baseline levels. The levels of this enzyme were significantly different between the 4 groups ($P < 0.01$, ANOVA), and were significantly higher in each of the treatment groups than in the control group ($P < 0.05$, Fisher's test). The induction of stromelysin in parallel with collagenase by β-estradiol, relaxin, or β-estradiol and relaxin is in agreement with the known coordinate regulation of these proteinases (Chin et al. 1985; Frisch et al. 1987; Birkedal-Hansen et al. 1993; Kapila et al. 1996).

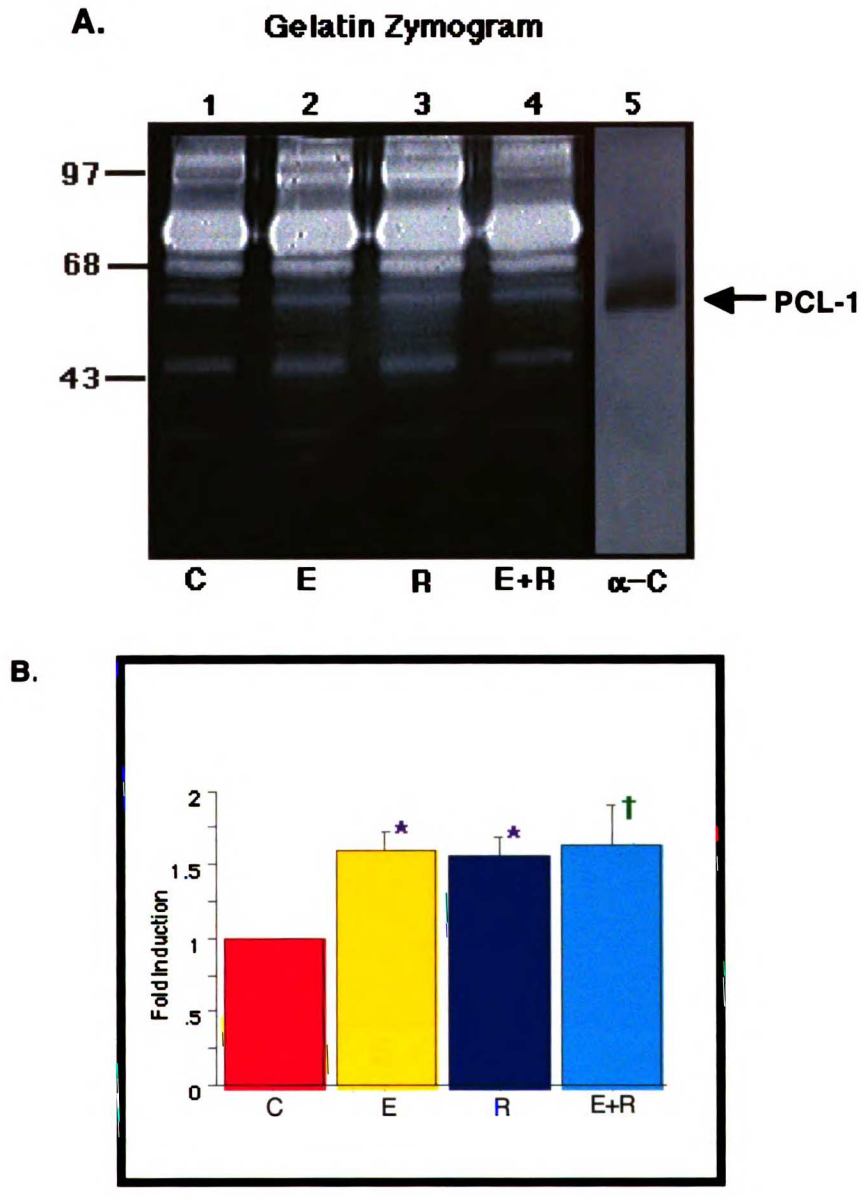

Figure 3: Induction of collagenase by β-estradiol, relaxin, and β-estradiol plus relaxin. Discs were incubated in 150 μl of serum-free medium alone (C; control) or in the presence of β-estradiol (E; 20 ng/ml), relaxin (R; 0.1ng/ml), or β-estradiol plus relaxin (E+R) for 48 hours. The disc-conditioned medium, standardized by total disc weight (mean wt .013 mg, SE ± .009), was assayed by gelatin substrate zymography (A). The 53/58-kDa gelatinolytic proteinase was identified in a selected sample to be procollagenase-1 (proMMP-1, PCl-1, lane 5) by Western immunoblot using a monoclonal antibody to rabbit collagenase (αC). The levels of 53/58-kDa gelatinolytic proteinases (procollagenase) were determined by scanning video-densitometry of gelatin zymograms from nine separate animals (36 disc sections). The mean (± S.E.) fold-induction of this proteinase by E, R and E+R relative to control baseline levels were determined and plotted as a histogram (B). Statistical analysis revealed significant differences in the 53/58-kDa proteinase between the 4 groups ($P < 0.05$, ANOVA) which arose from significantly higher levels of this enzyme in E or R treatments (*$P < 0.05$, Fisher's test) and E+R treatment (†$P < 0.01$) over control baseline levels. Molecular mass standards (in kilodaltons) are shown to the left of the gel.

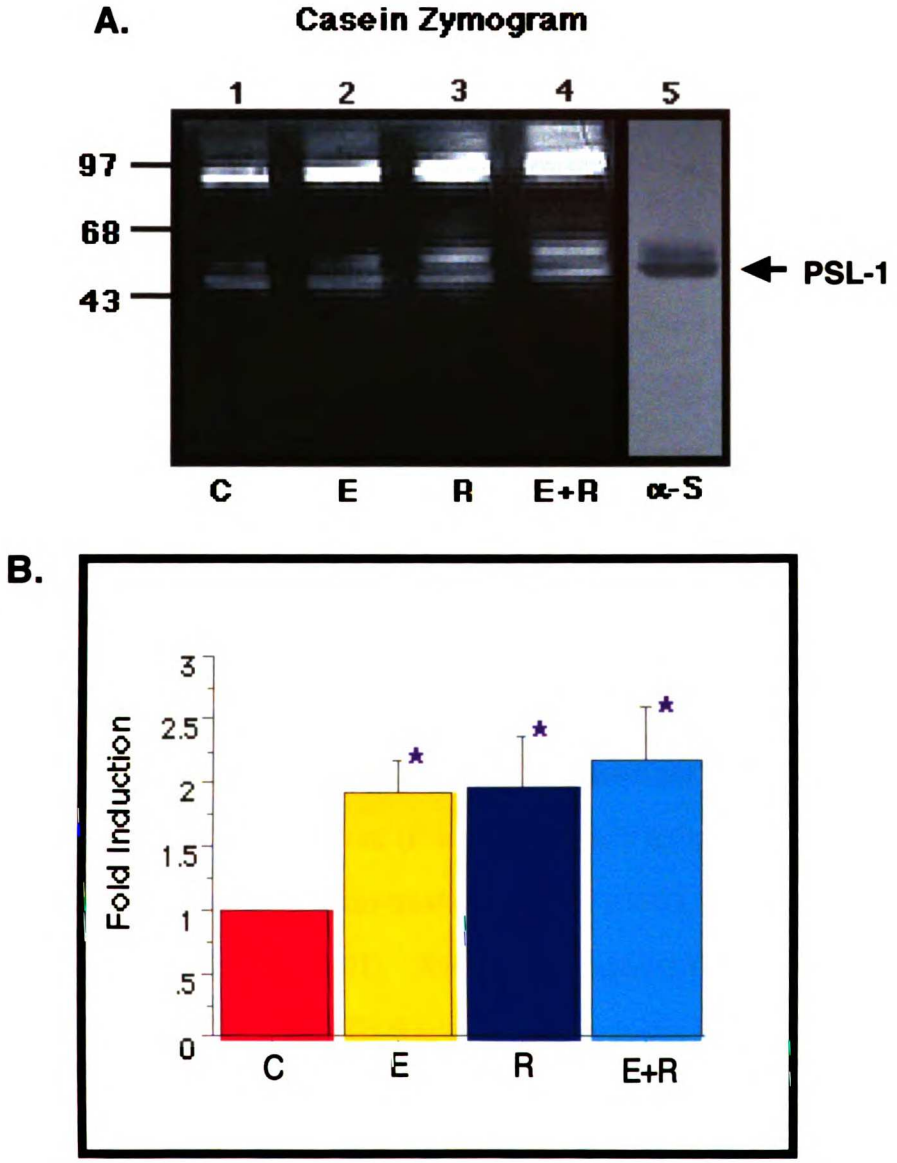

Figure 4: Induction of stromelysin by β-estradiol, relaxin, and β-estradiol plus relaxin. Discs were incubated in 150 μl of serum-free medium alone (C; control) or in the presence of β-estradiol (E; 20 ng/ml), relaxin (R; 0.1ng/ml), or β-estradiol plus relaxin (E+R) for 48 hours. The disc-conditioned medium, standardized by total disc weight (mean=.013 mg, SE ± .009), was assayed by casein substrate zymography (A). The 51/54-kDa caseinolytic proteinase was identified in a selected sample to be prostromelysin-1 (proMMP-3, PSI-1, lane 5) by Western immunoblot using a monoclonal antibody to stromelysin-1 (αS). The levels of 51/54-kDa caseinolytic proteinases (prostromelysin) were determined by scanning video-densitometry of casein zymograms from nine separate animals (36 disc sections). The mean (± S.E.) fold-induction of this proteinase by E, R, and E+R relative to control baseline levels were determined and plotted as a histogram (B). Statistical analysis revealed significant differences in the 51/54-kDa proteinase between the 4 groups ($P < 0.01$, ANOVA) which arose from significantly higher levels of this enzyme in E or R or E+R treatments (*$P < 0.05$, Fisher's test) over control baseline levels. Molecular mass standards (in kilodaltons) are shown to the left of the gel.

Relaxin and Relaxin plus β-estradiol Reduce the Area of the Disc Staining for Glycosaminoglycans

Since treatment of disc explants with E, R or E+R induced stromelysin and 92-kDa gelatinase, we next examined if the increase in levels of these proteinases was accompanied by loss in one of their substrates, the proteoglycans, from the disc. We made a direct assessment for the changes in proteoglycan content in the disc by staining disc sections from seven animals (28 disc sections) for GAGs using Safranin-O. Representative sections of the disc from the entire depth of disc were analyzed for GAG staining (Fig. 5A). Histomorphometric quantitation of Safranin-O staining of the TMJ disc demonstrated that, on average, 30.1% (S.E. 2.8%) and 29.7% (S.E. 4.7%) of the total disc area in control and β-estradiol-treated discs, respectively, stained positive for GAG's (Fig. 5B). In contrast, only 19.2 % (S.E. 3.3%) of the area of the disc in the relaxin-treated group and 16.9% (S.E. 2.7%) in the E+R-treated group stained positive for GAG's. These findings reflect statistically significant differences ($P < 0.02$, ANOVA) in GAG staining between discs from the control versus the relaxin-treated group ($P < 0.03$, Fisher's test) and control versus the E+R-treated group ($P < 0.01$). Similarly, compared to the estrogen group, the proportion of the disc staining for GAGs was significantly lower in the relaxin-treated ($P < 0.05$, Fisher's test) and E+R-treated ($P < 0.02$) groups.

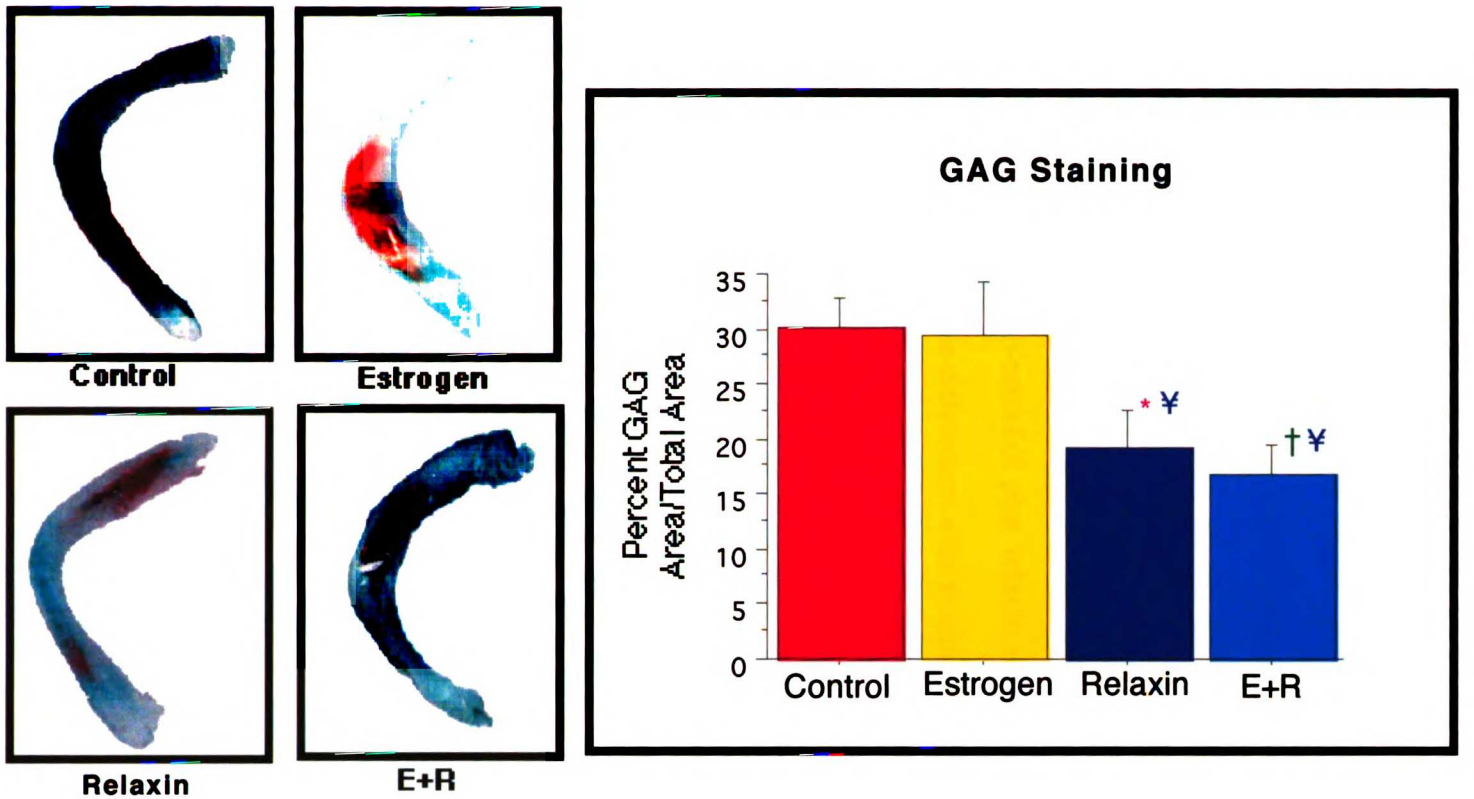

Figure 5: Exposure of discs to relaxin and β-estradiol plus relaxin reduces the amount of GAGs. (A) Safranin-O staining of representative sections of discs cultured in control serum-free medium, or in medium containing 20 ng/ml of β-estradiol, or 0.1 ng/ml or relaxin or β-estradiol and relaxin. The green area represents the counter stain (fast green), while the red area represents GAG staining (images were scanned into Photoshop at 1x magnification and then magnified 100% in Photoshop). The mean (± S.E) ratio of area staining for GAGs to total area of the disc was derived from seven animals (28 disc sections) and plotted as a histogram (B). Statistical analysis revealed an overall significant difference in the proportion of the disc staining positive for GAGs ($P < 0.02$, ANOVA). Intergroup comparisons showed that the relaxin-treated discs had significantly reduced area of GAG staining than control (*$P < 0.03$, Fisher's test) and the β-estradiol-treated (¥$P < 0.05$) groups. Similarly, the E+R-treated discs had significantly reduced areas of GAG staining relative to control (†$P < 0.01$) and β-estradiol-treated (¥$P < 0.02$) groups. Abbreviations are: E+R, β-estradiol plus relaxin.

β-estradiol Enhances the Synthesis of GAGs

Because the induction of stromelysin by relaxin and β-estradiol plus relaxin was associated with decreased GAG staining, while the induction of this MMP by β-estradiol was not accompanied by any decrease in GAG staining, we next determined if the disparity of the findings in β-estradiol-treated discs could be attributed to this hormone's modulation of GAG synthesis. Using ^{35}S we found that compared to the baseline control levels, discs exposed to β-estradiol showed increased levels of GAG synthesis in nine separate animals (36 disc sections) with a mean weight of .014 mg (SE ± .001). Similarly, discs exposed to relaxin had slightly elevated levels of GAG synthesis relative to control discs, while exposure of discs to β-estradiol plus relaxin resulted in no change in GAG synthesis. However, none of these differences were statistically significant.

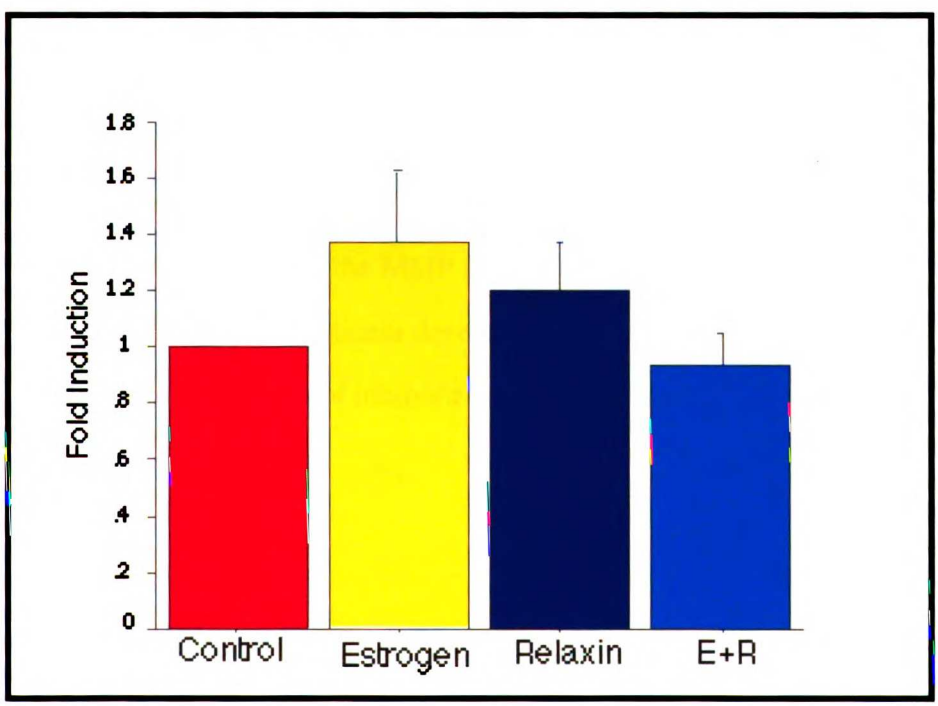

Figure 6: Modulation of GAG synthesis by β-estradiol and relaxin. GAG synthesis was determined by incorporation of ^{35}S by the disc explants. Discs were incubated with 0.0044 mCurie of ^{35}S in control serum-free medium or medium containing 20 ng/ml β-estradiol or 0.1 ng/ml relaxin or β-estradiol plus relaxin for 6 hours. The discs were then digested with papain for 12 hours. The radioactivity in 25 μl aliquots of the digest was measured and standardized to total disc weight. The mean (±S.E.) fold change in ^{35}S incorporation for the three hormone treatments relative to control baseline levels was derived from nine separate animals (36 disc sections) and plotted as a histogram. Abbreviations are: E+R, β-estradiol plus relaxin.

Preliminary Studies on the Effects of MMP Inhibitor on MMP Activity and GAGs in TMJ Disc Explants

To examine the effects of the relaxin- and β-estradiol-induced MMPs on disc GAGs or proteoglycan content, the synthetic metalloproteinase inhibitor GM6001 or its control synthetic analogue was used in explant cultures. An additional purpose of this preliminary study was to determine the appropriate range of concentrations of inhibitors for these experiments. For this preliminary study, disc halves from six separate animals with a mean weight of .013 mg (SE ± .006) were exposed to control media in addition to the different hormone treatments as described previously in the presence of 100 μM of GM6001 inhibitor (3 animals, 12 disc sections), or its control analogue (3 animals, 12 disc sections). Analysis of disc-conditioned media by gelatin substrate zymograms demonstrated that GM6001 inhibitor caused substantial reduction in the activities of the 92-kDa, 72-kDa and 53/58-kDa gelatinolytic proteinases over that observed in discs cultured with the control analogue (Fig. 7A). Similarly, the casein substrate zymograms demonstrated a GM6001 inhibitor-mediated decrease in the activity of the 51/54-kDa caseinolytic proteinase (Fig. 7B). However, since the MMP inhibitor also decreased the activity of the constitutively expressed MMPs, additional dose-response studies need to be conducted to determine the optimal concentration of inhibitor for our experiments.

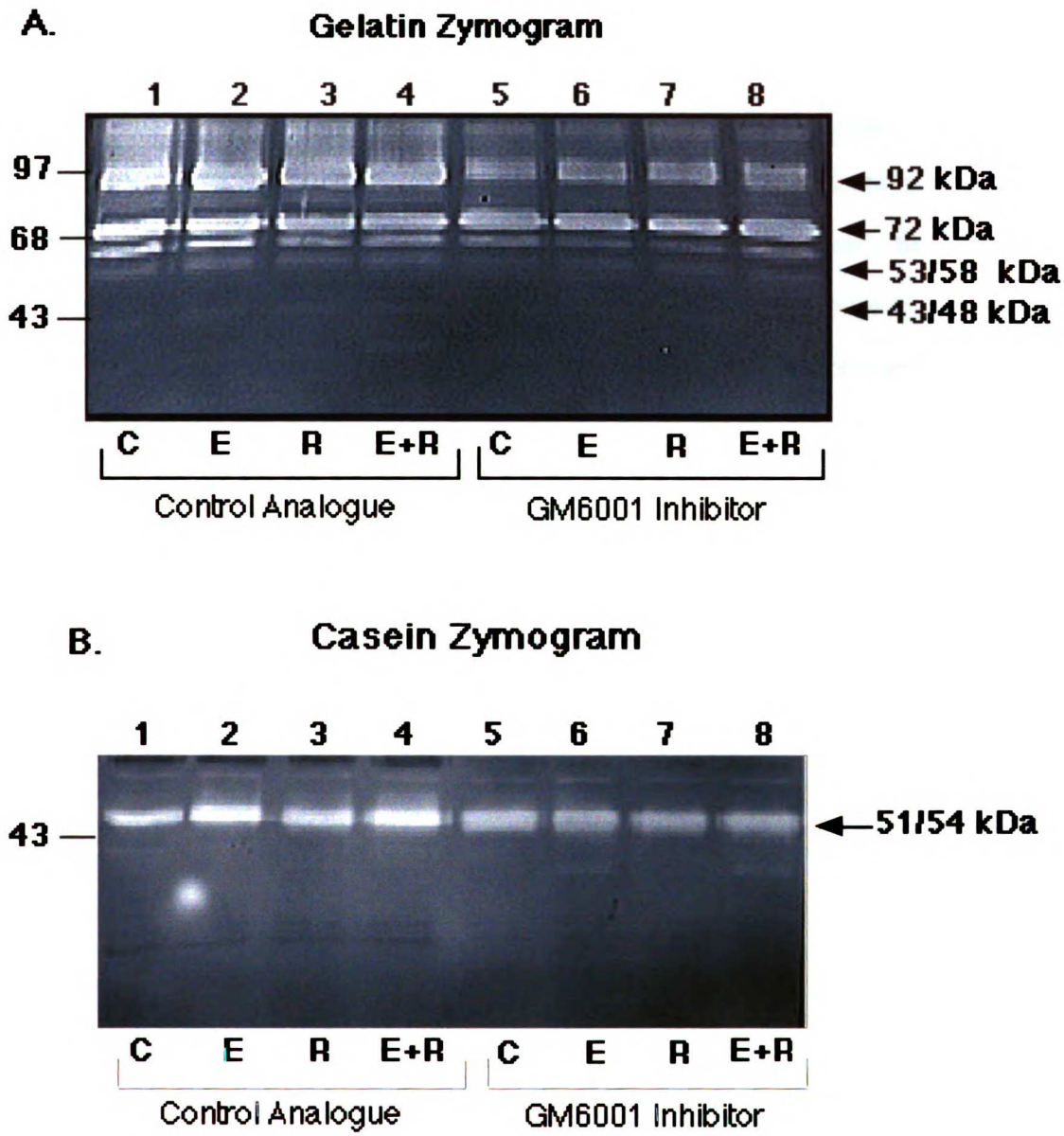

Figure 7: Modulation of gelatinolytic and caseinolytic proteinase activities from six separate animals (3 control analogue + 3 inhibitor) by the GM6001 inhibitor and its control analogue. Discs were incubated in serum-free medium alone (C; lanes 1 and 5) or in medium containing 20 ng/ml of β-estradiol (E; lanes 2 and 6) or 0.1 ng/ml of relaxin (R; lanes 3 and 7) or β-estradiol plus relaxin (E+R; lanes 4 and 8) in the presence of the control analogue (lanes 1 to 4) or GM6001 inhibitor (lanes 5 to 8). The disc-conditioned medium was assayed by gelatin substrate zymography (A) or casein substrate zymography (B). The MMP inhibitor produced a generalized reduction in all gelatinolytic and caseinolytic proteinase activities. Molecular mass standards in (kilodaltons) are shown to the left of the gel.

To examine if the increased expression of MMPs in response to relaxin and β-estradiol plus relaxin are directly responsible for the net loss of disc GAGs, the effects of the synthetic inhibitor (GM6001) on disc GAG or proteoglycan content was also determined. Our preliminary studies from six animals (18 disc sections) on disc digests showed that compared to the baseline control analogue (3 animals), the total amount of proteoglycans in the discs were generally increased in all of the groups exposed to 100 μM of GM6001 inhibitor (3 animals) (Fig. 8). Additionally, histomorphometric quantitation of four Safranin-O stained disc halves from one animal exposed to medium with or without hormones and 100 mM of GM6001 inhibitor demonstrated an approximately equal percent of disc area staining positive for GAGs in control (24.9% ±S.E. 1.6%) and all hormone treatments (β-estradiol = 24.6% ±S.E. 2.2%; relaxin = 26.2% ±S.E. 1.2%; β-estradiol + relaxin = 25.4% ±S.E. 1.5%) (Fig. 9). Therefore, as opposed to the relaxin- and β-estradiol plus relaxin-mediated loss in GAGs in the absence of the inhibitor (see Fig. 5), in the presence of the inhibitor the discs in the three hormone treatment groups showed similar GAG staining to each other and the control group. However, all these findings are preliminary and require refinement of protocol, determination of the appropriate concentration of the inhibitor to be used and an increase in sample size before any definitive conclusions can be made.

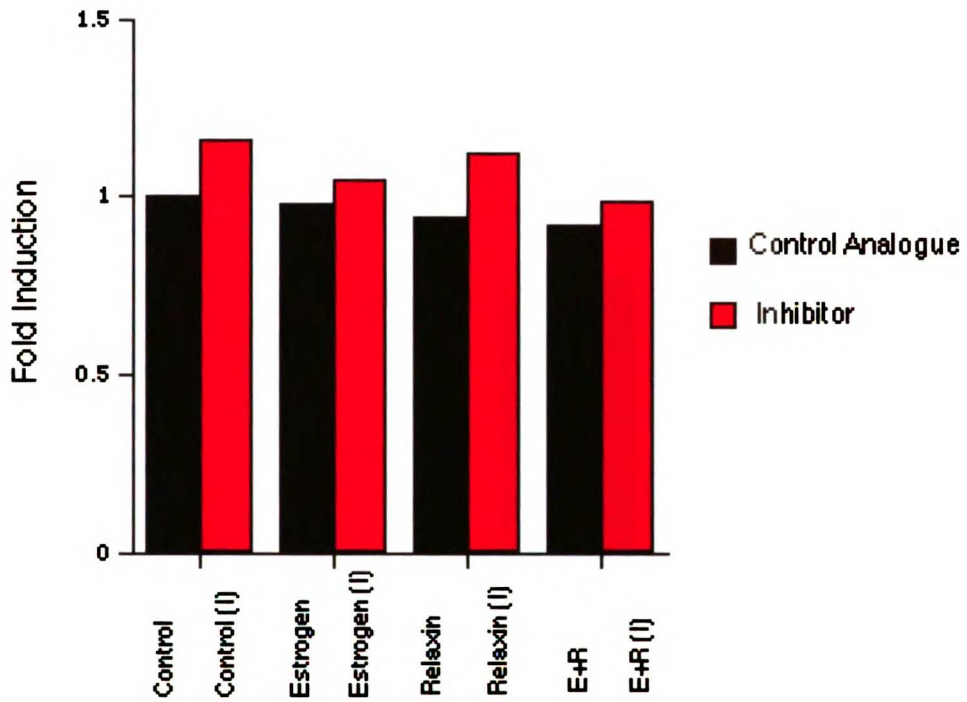

Figure 8: Effects of the MMP inhibitor GM6001 on proteolgycan content in discs exposed to different hormone treatments. Discs were incubated as described in figure 3 with the addition of 100 μM of either of the inhibitor GM6001 (3 animals) or its control analogue (3 animals). The discs were digested with papain, and the proteoglycan content in the discs were measured using the DMB binding assay and standardized to total disc weight. The fold change in proteoglycan content for the three hormone treatments in the presence of inhibitor relative to control baseline levels with the control analogue group was derived and plotted as a histogram. Relative to the baseline control group with the control analogue, the levels of total proteoglycan content in the discs were slightly increased in the presence of inhibitor. (I = inhibitor).

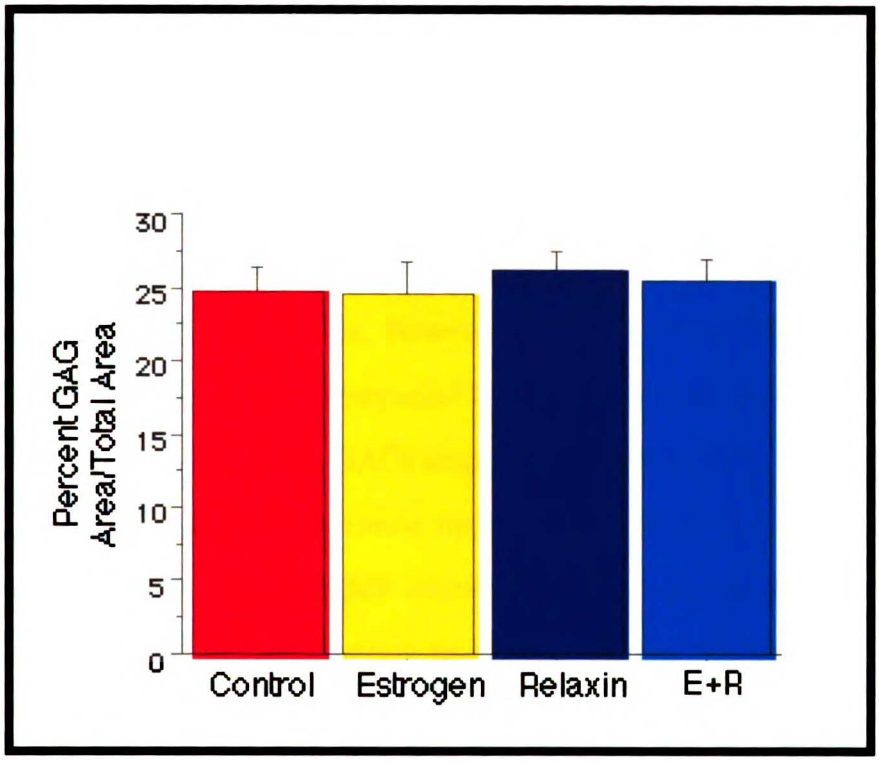

Figure 9: Effects of the MMP inhibitor GM6001 on disc GAGs from one animal (4 disc sections). Discs were cultured in control serum free media or media containing β-estradiol, or relaxin or β-estradiol plus relaxin (E+R) as described in figure 3. Additionally, 100 mM of GM6001 was added to each of the explant cultures. After 48 hours of culture, the discs were fixed, embedded, sectioned and stained for GAGs. The mean (±S.E.) of approximately 10 sections from each of the treatment groups of one experiment for the ratio of area staining for GAGs to total area of the disc was derived and plotted as a histogram. No difference in the area of GAG staining between the control group and all treatment groups was noted. The area of GAG staining in the relaxin and β-estradiol plus relaxin groups on average were increased compared to discs incubated in the absence of the inhibitor (refer to figure 5).

IV. DISCUSSION

This study demonstrates that β-estradiol, relaxin and β-estradiol plus relaxin all induce increased expression of collagenase-1 and stromelysin-1 in rabbit TMJ disc explants. The relaxin and β-estradiol plus relaxin-mediated increases in these MMPs were paralleled by a loss in disc GAGs. However, induction of collagenase-1 and stromelysin-1 by β-estradiol alone was not accompanied by changes in GAG staining within the disc. β-estradiol's maintenance of disc GAGs despite its modulation of increased MMP expression could have resulted from this hormone mediating an increase in GAG synthesis. Further, preliminary studies using the MMP inhibitor, GM6001 indicate that decreased activity of hormone-induced MMPs may prevent loss of disc GAGs. However, these findings need to be confirmed through more definitive studies. Together, these studies demonstrate that relaxin, with or without β-estradiol, induces MMP expression that are accompanied by an altered matrix composition in TMJ disc explants. The findings therefore implicate these hormones in a subset of TMJ diseases in women.

While the induction of collagenase-1 and stromelysin-1 by relaxin and β-estradiol plus relaxin in disc explants showed similarities to that observed in isolated disc cells in culture (Kapila and Xie 1998), some differences in the responses were also observed. These included the lack of potentiation by β-estradiol of relaxin's induction of MMPs which was observed in the previous study, and the induction of MMPs by β-estradiol which did not occur in isolated cells. While the studies on disc explants are likely to be more physiologically relevant than those on isolated cells in culture, it is important to understand the reasons for the differences between the findings of the two studies. Several potential reasons may explain the differences observed between the responses of isolated cells and cells in disc explants to these hormones. The most likely of these include the dedifferentiation and altered phenotype as well as the loss of the natural matrix environment of cells in culture versus those in explants.

It is commonly known that cells in culture undergo dedifferentiation and therefore are likely to respond differently than cells in intact tissues. Thus chondroyctyes in culture lose their cartilaginous phenotype, become dedifferentiated, exhibit a fibroblastic morphology, and synthesize low molecular weight proteoglycans (Liu et al. 1998). The progression of cells toward dedifferentiation may be accompanied by a change in their constitutively expressed MMPs (Grant et al. 1996; Nawrock et al. 1997; Lemare et al. 1998), as well as changes in the regulation of MMP expression by exogenous agents (Neff et al. 1982; Erickson et al. 1997; Nawrock et al. 1997; Lemare et al. 1998; van Osch et al. 1998). Thus, for example, the expression of 72-kDa gelatinase and stromelysin-3 mRNA increases progressively whereas TIMP expression decreases with dedifferentiation in stromal cells (Nawrock et al. 1997). Furthermore, chondrocytes in culture show an impaired 92-kDa gelatinase response to interleukin-1β, that can be recovered after restoring the differentiated phenotype of the chondrocytes with alginate beads (Lemare et al. 1998). Similarly, rabbit articular chondrocytes demonstrate varied morphology, proteoglycan content and type of collagen in the presence of TGF-β depending on the differentiation stage of the cells (van Osch et al. 1998). Although our previous studies on isolated disc cells and disc explants demonstrate their similar repertoire of constitutively expressed MMPs (Kapila et al. 1995), the effects of cell culture on the differentiation and responsiveness of disc cells to exogenous agents including β-estradiol and relaxin require further study.

Either related or unrelated to the effects of culturing cells on their differentiation status, the extracellular matrix environment may also alter the response of cells to exogenous agents. Since using TMJ disc explants preserves an intact interacting network of matrix macromolecules, it represents a complex and rather different environment compared to isolated cells in culture. The extracellular matrix and the intricate collagen network present in the intact tissues is rich in information not only because it is comprised of multifunctional structural ligands for cell surface receptors, but also because it contains

signaling factors (Damsky et al. 1997). Thus, many matrix molecules and matrix-bound growth factors serve as receptors and mediators of cell function and regulation both in solid and soluble forms. These cell-matrix interactions contribute to several normal cellular functions including proliferation, differentiation, locomotion and metabolic activities (Gospodarowicz and Ill 1980; Toole 1991), and also alter responses of the cells to exogenous agents. Additionally, cells in culture may express variant or different isoforms of matrix macromolecules than they express in intact tissues (Burton-Wurster et al. 1998), thereby further increasing the disparity between their natural and in vitro matrix environments. Thus, the functions and interactions of the groups of molecules present in the intact disc are complex, extensively interrelated and may not only effect differentiation of cells, but also their MMP responses to the hormones.

The dedifferentiation of cells in culture or their altered extracellular matrix environment may modify their responses to exogenous agents due to changes in the profiles, binding capacity or number of receptors expressed (Dharmavaram et al. 1997; Monsonego et al. 1997), or due to other changes within the cells. Thus, for example it has been shown that cultured chondrocytes from avian growth-plates undergo dedifferentiation that is accompanied by a reduction in their response to growth hormone due to the differentiation-dependent loss of the extracellular domain of the growth hormone receptor (Monsonego et al. 1997). Furthermore, it has also been demonstrated that DNA binding activity of the Sp1 transcription factor differs between differentiated and dedifferentiated chondrocytes (Dharmavaram et al. 1997). These studies provide evidence that variation in status of cellular differentiation is accompanied by changes in the intrinsic functions of the cell that could explain the observed differences in response of the cells in disc explants from those of isolated disc cells to relaxin and β-estradiol. However, further studies including those assessing the potential changes in relaxin and β-estradiol receptors in differentiated versus dedifferentiated disc cells are recommended in order to address these questions.

For the reasons cited above, cells in disc explants may be more responsive to relaxin than those in culture such that β-estradiol may not potentiate their response to relaxin. This postulate is made on the basis of our previous findings (Kapila and Xie 1998) showing that, while cells in culture demonstrate substantial potentiation of MMP induction by β-estradiol at 0.1 ng/ml or less of relaxin, those exposed to 1 ng/ml or more of relaxin show only a slight potentiation of MMP induction by β-estradiol. It is plausible that cells in disc explants do not show a potentiation of MMP-induction by β-estradiol at 0.1 ng/ml of relaxin as do isolated cells, but may do so at lower concentrations. Future relaxin dose-response experiments in TMJ disc explants may provide further insights for the reasons for the lack of β-estradiol's potentiation of MMP induction by relaxin. An additional reason for the lack of β-estradiol's potentiation of MMP induction by relaxin in disc explants versus isolated cells could be that, while in the present study β-estradiol and relaxin were added concurrently to the explant cultures, in the previous study the cells were primed with β-estradiol prior to exposure to relaxin. This difference in temporal exposure of the hormones could also contribute to the variation in the observed responses.

Finally, β-estradiol's induction of collagenase in disc explants but not in isolated cells may be attributed to its ability to induce the collagenase-1 promoter in some cell types but not in other related cell types (Webb et al. 1995). Thus tamoxifen, an estrogen analogue, is a potent activator of estrogen receptor mediated induction of promoters regulated by AP-1 sites including the human collagenase gene promoter, and tamoxifen's agonism at AP-1 site has been shown to be cell-type specific. These findings may explain why any changes in phenotype or differentiation status on isolating the cells may result in changes in cellular responses to β-estradiol.

We also found that the induction of collagenase and stromelysin by relaxin or relaxin plus β-estradiol was paralleled by a loss in disc GAGs. In contrast, despite β-estradiol's induction of collagenase-1 and stromelysin-1, no loss in disc GAGs was noticed. This finding may be attributed to the increased synthesis of GAG as measured by

^{35}S incorporation in response to β-estradiol as compared to that in baseline control group as well as the relaxin and β-estradiol plus relaxin groups. However, the increase in ^{35}S incorporation in β-estradiol-treated discs was not statistically greater than in control discs or those exposed to other hormone treatments. Additional studies to increase the sample size are planned to clearly define the role that GAG synthesis plays in the disc matrix remodeling response to β-estradiol.

That the relaxin- or relaxin plus β-estradiol-induced MMPs may be directly responsible for the loss of disc GAGs was suggested by our preliminary studies using the synthetic MMP inhibitor, GM6001. We found that the inhibitor at concentrations as low as 100 μM reduced the levels of gelatinolytic and caseinolytic MMPs expressed by disc explants. Additionally, the proteoglycan concentration in disc digests as well as the GAG staining in discs were generally greater in discs exposed to the MMP inhibitor then to its control analogue. However, these were only minor trends and do not provide definitive findings on an association between MMP induction by relaxin (± β-estradiol) and disc GAG loss. Further refinements in the experimental protocol will help us better address this hypothesis. Furthermore, our findings together with other studies (Leppert et al. 1995; Chin and Werb 1997) using GM6001 suggest that future studies should be directed at determining the optimal inhibitor concentration which would produce the appropriate levels of inhibition of hormone-induced MMPs.

Clinical Relevance

Our findings on the induction of MMPs and GAG loss by relaxin with or without β-estradiol implicate the role of these hormones in a subset of TMJ disease in women. By modulating the expression of MMPs and matrix macromolecules, relaxin and estrogen may cause or predispose to joint disease by at least two mechanisms. First, since relaxin affects the composition of collagen (Chihal and Epsey 1973), and induces collagenase and stromelysin in ligaments (unpublished data), it can increase the laxity of the supporting

structures of the joint, leading to hypermobility and subsequent osteoarthritis. This mode of action is likely to affect most joints and may be one of the mechanisms by which systemic joint hypermobility occurs in women. Secondly, a tissue relatively unique to the TMJ, specifically the disc, may serve as a specific target for the matrix remodeling actions of relaxin and estrogen. The latter mechanism may explain the distinct age and gender distribution of diseases of other joints.

The findings of the present study also have an implied physiologic relevance because the induction of collagenase-1 and stromelysin-1 occurred at concentrations of relaxin found systemically in cycling women (Eddie et al. 1986; Bell et al. 1987). However, whether systemic relaxin can access the TMJ to reach the disc remains to be determined. Because the TMJ disc is an avascular tissue bathed in synovial fluid, its response to relaxin with or without estrogen is likely a local factor, rather than systemic. Although there is little evidence for the presence of estrogen and relaxin in synovial fluid from healthy joints, estrogen has been detected in synovial fluid of osteoarthritic joints (Tsai et al. 1992). Access of systemic factors and nutrients to cells of the TMJ disc occur via the synovium attached to the periphery of the disc, and through the synovial fluid in which the disc is bathed. The synovial fluid in healthy tissues maintains a composition substantially different from that of serum because the synovial lining has a selectively permeable membrane which also secretes specific proteins and proteoglycans into the synovial fluid. In contrast, during inflammation the composition of the synovial fluid becomes similar to serum (Harris 1989), with the possibility that systemic factors such as relaxin may reach the disc in sufficient concentrations to further aggravate tissue degradation. Molecules of up to 10-kDa can diffuse passively across healthy blood vessels (Andries and Brutsaert 1994), and contrast media of at least 92-kDa can traverse through the walls of hyperpermeable vessels in inflamed joints (van Dijke et al. 1997). It is probable that the small 6-kDa relaxin and the 356-Da β-estradiol may diffuse unimpeded through the healthy or diseased synovial tissues into the joint space. Furthermore, if

systemic relaxin can access the disc, it is important to know if this is at sufficient concentrations to produce the matrix-degradative response as observed in explanted discs in culture. Our findings suggest a mechanism by which female reproductive hormones may predispose women of reproductive age to joint matrix degradation, particularly in the TMJ and other joints with a large proportion of fibrocartilaginous tissues. However, additional animal and clinical studies are needed to further clarify the role of relaxin, with and without the modulatory effects of estrogen, in inducing the degradative remodeling of the TMJ disc.

Finally, preliminary studies with the GM6001 synthetic inhibitor demonstrated its inhibitory effects on the gelatinolytic and caseinolytic MMPs at concentrations as low as 100 μM. These findings not only provide insights into the potential role of increased matrix degradation in tissue loss during joint disorders, but also suggest potential use of MMP inhibitors as therapeutic agents. However, it should be noted that although several synthetic peptides have been shown to be potent inhibitors of MMPs *in vitro*, their biostability currently limits their usefulness clinically. Perhaps the greatest difficulty lies in achieving therapeutic concentrations *in vivo*. To effectively decrease cartilage breakdown, metalloproteinase inhibitors must enter the cartilage, which means they must penetrate the dense network of collagen and proteoglycan aggregates. These aggregates are highly negatively charged and this, in turn may further impede entry of an inhibitor. Further design modifications in synthetic MMP inhibitors is likely to improve their utility in various degenerative diseases.

Future Studies

The findings from these studies provide insights into the potential hormonal etiology of TMJ disease in women and the mechanisms by which specific female reproductive hormones may initiate or aggravate TMJ diseases. These studies lay the foundation for future studies that should address the characteristics of relaxin-binding to the TMJ disc and ligaments cells, the dynamics of access of relaxin into the TMJ, and

quantitatively and qualitatively evaluate the loss of matrix macromolecules in response to relaxin. While these studies may indicate the reasons for the age and gender distribution of TMJs, ongoing studies on ligaments may provide insights into how relaxin, with or without modulation by estrogen, may predispose to systemic joint laxity and development of disease in other joints. The findings of these studies may be critical in designing specific diagnostic methods, and biologically rational therapeutic and preventive approaches for TMJ disease in women.

Other important questions that need to be addressed include: (1) Can hormones such as estrogen and relaxin initiate these diseases or do they aggravate an existing disease state? (2) Can these hormones access the healthy TMJ or do they require the presence of inflammation? Answers to these questions will help us to identify or distinguish between initiating factors or agents that aggravate existing joint pathology. The potential role of relaxin in initiating or aggravating TMJ disease can also be evaluated by utilizing an animal model of TMJ arthritis previously developed in our laboratory (Kapila et al. 1995). Use of this model with mild levels of arthritis could help to determine whether low-grade inflammation alters the ability of relaxin to access the TMJ, particularly the disc, and will be useful in delineating the potential role of relaxin in perpetuating joint diseases initiated by inflammation caused by acute trauma or exogenous agents. These *in vivo* studies should also be used to determine whether systemic relaxin affects expression of MMPs and TIMPs, matrix synthesis, and ultimately the matrix composition of the TMJ disc. Additionally, future studies examining the levels of collagen in the disc will determine the effects of increased collagenase expression on the collagen content in the disc.

An additional aspect in understanding the biology of relaxin is to identify its sites of action through localization of relaxin receptors. While the receptor (s) to relaxin have yet to be sequenced and cloned, use of radiolabeled relaxin has revealed the presence of high affinity relaxin-binding molecules on cells in the fibrocartilaginous pubic symphysis (Samuel et al. 1996). This together with our previous and present studies demonstrating an

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