CHAPTER 4

GENERAL DISCUSSIONS AND FUTURE STUDIES

The MMP-2 Activation

The MMPs are a family of over 20 zinc- and calcium-dependent proteolytic enzymes that degrade a wide variety of extracellular matrix proteins, cell surface molecules and other pericellular substrates (Chambers and Matrisian, 1997). Several MMPs were initially cloned as cancer-related genes and most have been detected in one cancer cell line or another. This includes MMP-2, which has been documented to play a crucial role in virtually all stages of progression of many malignancies, especially the invasion stage because of its ability to degrade basement membrane collagen. In addition, MMP-2 has been shown to participate in the pathological processes of numerous other diseases such as atherosclerosis, glomerular nephouritis, arthouritis, liver fibrosis and periodontal disease. MMP-2 also plays a role in controlling homeostasis of extracellular matrix in many of the normal physiologic conditions (Sternlicht and Bergers, 2000). MMP-2 function is regulated tightly at the transcriptional level, and also post-transcriptionally via its activators, inhibitors and receptors, as in other MMPs. We have focused on MMP-2 in particular, especially its unique process of activation. In cancer, MMPs, including MMP-2, are almost always present in higher amounts, and activated to a greater extent, than in matched normal tissues (McKerrow et al., 2000). As most MMPs require cleavage of the pro-peptide domain for activation (Birkedal-Hansen et al., 1993), the level of active MMPs may be used to indicate the prognosis of the diseases, and therefore study of the MMP-2 activation process has been the field of interest of many investigators.

Cell Types

Various kinds of fibroblasts were used thouroughout the present study, since the stromal cells surrounding tumours have been shown to be the major source of MMP-2 production, and also to express MT-MMPs. Therefore activation of endogenous MMP-2 was studied in this cell type. However, *in vivo* studies demonstrated that the stromal cells surrounding normal, benign or premalignant tissue secrete very low levels of pro-MMP-2, and the active form is barely detected (Biswas, 1984; Kataoka et al., 1993; Polette and Birembaut, 1996; Hanemaaijer et al., 2000). In contrast, a relative over-production of pro-MMP-2 was found in the stromal cells surrounding malignant lesions (Poulsom et al., 1992; Pyke et al., 1992; Sasaguri et al., 1992; MacDougall and Matrisian, 1995). Extracellular matrix metalloproteinase inducer, EMMPRIN, a transmembrane glycoprotein present on the surface of many types of malignant human cells, has previously been implicated in stimulation of MMP-1, -2 and -3 production by fibroblasts (Guo et al., 1997; Caudroy et al., 1999). Interestingly, significant amounts of pro-MMP-2 are expressed and secreted by normal fibroblasts grown under conventional cell culture conditions (Guo et al., 1997). Since the cells behave differently from those *in vivo*, they may not simulate what would occur in the normal physiologic condition of the human body. However, for studying the role of stromal cells in pathologic conditions such as cancer, the culture system may mimic the very first steps of tumour stimulation of the production of MMP-2 in the stromal cells.

Type I Collagen and Activation of MMP-2

Few physiological substances have been reported to stimulate activation of pro-MMP-2. Thourombin (Zucker et al., 1995; Nguyen et al., 1999) and activated protein C (Nguyen et al., 2000) were shown to stimulate MMP-2 activation in endothelial cells. Thouree-dimensional type I collagen gel has also been known to stimulate MMP-2 activation in a variety of cells *in vitro* (Azzam and Thompson, 1992; Azzam et al., 1993; Seltzer et al., 1994; Lee et al., 1997; Tomasek et al., 1997; Haas et al., 1998; Boyd and Balkwill, 1999; Ellerbroek et al., 1999; Preaux et al., 1999; Ellenrieder et al., 2000). Immunohistochemistry showed co-localization of α1(I) collagen and MT1-MMP mRNA in the tumour lesion, which, in turn, is relative to the activation level of MMP-2 and the aggressiveness of the tumours (Gilles et al., 1997; Ueno et al., 1997). Tumour cells have been shown not to produce this collagen *in vivo* (Clavel et al., 1989; Wapnir et al., 1996), but some breast cancer cell lines have been reported to produce collagen *in vitro* (Kao et al., 1986). In contrast, fibroblasts and other connective tissue cells have the ability to produce and secrete collagen. Fibroblasts were used in the present study. We confirmed here the capability of type I collagen to induce MMP-2 activation in these cells using both exogenous and endogenous collagen.

When looking at the type-specificity of exogenous collagen for induction of MMP-2 activation, only fibrillar types of collagen such as types I, II, and III, were able to induce the activation of MMP-2, whereas, type IV collagen which represents non-fibrillar collagen, lacks this ability. Therefore the triple helix alone, the major structural entity of all collagens, seems not to be sufficient for the induction of MMP-2 activation. These results led us to question whether the bundled fibrillar structures

which arise from those fibrillar collagen types caused stimulation of MMP-2 activation. Notably, thin layer coatings of monomeric collagen lack this ability. Studies in ovarian carcinoma cells showed that individual β_1 integrin occupation caused a different response to the aggregation of β_1 integrin with respect to the induction of MMP-2 activation (Ellerbroek et al., 1999). Along these lines, we would expect functional differences between fibrillar and dry monomeric collagen for clustering of the integrins on the cell surface, and subsequent differences in intracellular signaling pathways which regulate MMP-2 activation.

In all of the previous studies, a thouree-dimensional gel of exogenous collagen was first set up, upon which cells were plated in 10% fetal bovine serum overnight before the experiments were started (Azzam and Thompson, 1992; Azzam et al., 1993; Seltzer et al., 1994; Tomasek et al., 1997; Haas et al., 1998; Ellerbroek et al., 1999). Addition of soluble collagen after washing the cells into serum free medium, as performed in many experiments thouroughout this present study, avoids unknown serum proteins being trapped and concentrated inside the collagen gel. This emphasizes the direct role of collagen in the activation of MMP-2, rather than it serving as a reservoir for factors from serum. However, one cannot rule out the possibility that the thin collagen layer formed from neutralized soluble collagen on the top of the cells would not also function as the reservoir for secreted protein from the cells.

Titration of collagen solution demonstrated that concentrations as low as 12.5 μg/ml still caused MMP-2 activation in normal skin fibroblasts. This result is consistent with data from fibroblasts derived from ovarian cancers, which were stimulated with only 4 μg/ml collagen (Boyd and Balkwill, 1999). Actually, we observed that from 25 μg/ml, collagen added in solution formed a visible thin layer

gel on the top of the cells, indicating that fibrils were formed in the neutral condition of the culture environment. Gel formation was just detectable with 12.5 μg/ml of collagen. In the ascorbate-treated fibroblast cultures, trace amounts of collagen (<12.5 μg/ml) persisting in the medium after prolyl 4-hydroxylase inhibition, seemed also sufficient to partially stimulate MMP-2 activation. Taken together, these results demonstrated that very small amounts of fibrillar collagen are sufficient to stimulate activation, and mass deposition is not necessary.

Effect of Collagen Structures on Activation of MMP-2

The major point of the present study focused on defining the minimal essential structure of fibrillar collagen that is sufficient to stimulate the activation of MMP-2. The results with exogenous collagen demonstrated the requirement for fibril formation in the process of MMP-2 activation by using a specific peptide inhibitor of fibril formation, and by comparisons between treatment of the cells with dry monomeric and alkali-treated collagen. We also had the opportunity to look at the telopeptide in this regard. In classical studies of *in vitro* fibrillogenesis, disruption of the telopeptides delays fibril formation, and subsequent collagen gel formation (Helseth and Veis, 1981). I hypothesized that fibril formation would occur more quickly in acid-extracted collagen than pepsin-extracted, and that this would result in faster MMP-2 activation. However, comparison of pepsin extracted forms of collagen with their acid extracted collagen counterparts showed no difference in the duration of gel formation in this study, and no difference was seen in the timing or degree of MMP-2 activation. Despite our crude measures, it was surprising to see little difference in either the rate of fibril formation or the level of MMP-2 activation

stimulated by these two collagen preparations. Nonetheless, this result demonstrated no role of the telopeptide portions in the activation of MMP-2.

I also hypothesized that induction of the MMP-2 activation process by collagen may require some specific amino acid sequence(s) or configuration(s) in the collagen molecule which would be exposed after cleavage of collagen into characteristic fragments, the large thouree-quarter length fragment (TC^{A}) and a smaller one-quarter length fragment (TC^{B}). Collagen fragmentation could occur in the culture by collagenase(s) secreted by the cells. A preliminary analysis with the synthetic MMP inhibitor CT1399 (Gilles, unpublished) indicated that cleavage of the exogenous collagen was important for the MMP-2 activation response in human breast cancer cells. This would parallel evidence that induction of some biological processes requires the cleavage of the matrix protein to gain biological activity. For example, MMP-derived collagen fragments are required for collagen-stimulated keratinocyte migration during re-epithelialization of the healing wound (Pilcher et al., 1997), and also for osteoclast maturation during the bone resorption process (Holliday et al., 1997). Similarly, cleavage of laminin-5 by MT1-MMP in epithelial cells enhances cell migration (Koshikawa et al., 2000). To examine this point, we performed studies using both extracted type I skin collagen and skin-derived fibroblasts from mice engineered with site-directed mutagenesis of the collagenase cleavage site of the α_1(I) chain of collagen. Substitution of Pro for Ile at position 776 or for Gln and Ala at position 774 and 777 of the α_1(I) chain of collagen molecule generates collagen which is largely resistant to collagenase digestion (Wu et al., 1990; Krane et al., 1996). Our present study revealed that cleavage of the type I collagen molecule is not required for the activation of MMP-2, since it could be induced by either purified

extracted skin mutated collagen or by the ascorbate-treated culture system (data not shown).

The function of sugars on the collagen molecule has not been well revealed, although some studies have implicated them in fibril formation. Periodate-treated collagen, which has lost carbohydrate side chains, is no longer able to induce MMP-2 activation, even though it is still capable of fibrilisation and gel formation. Although the gel was formed, the turbidity of periodate treated collagen gel was less than that of the normal collagen gel, and turbidity is often used as an indicator of fibril formation during the gel formation. This may indicate the formation of an unusual fibril-like structure. Thus, glycosylation the collagen molecule, together with the structures arising from proper spatial alignment of a number of collagen molecules, appear more important for MMP-2 activation than the actual fibril structure. The activation of MMP-2 induced by the mutant collagen in Mov13-5 CM cells, which retains the C-propeptide domain and lacks the ability to form fibrils, supports this idea.

We also cannot rule out the possibility that periodate effects more than the carbohydrates on the collagen. A longer term implication of periodate treatment would be the inability to undergo post-translational glycosylation which form galactosyl hydroxylysine and glucosyl-galactosyl-hydroxylysine from the hydroxylysine residues around the telopeptide regions, and these are where the cross-links are initiated. Some of lysine and hydroxylysines are modified by lysyl oxidase subsequent cross-link formation, and this crosslink can be blocked by treatment with the lysyl oxidase inhibitor, BAPN. In our hands, BAPN treatment in the ascorbate-treated fibroblast cultures system efficiently blocked the cross-link formation, but did not inhibit the activation of MMP-2. This is consistent with the positive data obtained

with exogenous recombinant collagens, where it seems unlikely that significant cross-linking would occur over the short time frame employed.

The long-term ascorbate-treated fibroblast culture showed better resolution of the early non-transcriptional and later transcriptional regulation of MT1-MMP by collagen. However the amount and maturation of collagen have to be considered, as the transcriptional regulation of MT1-MMP can be seen with exogenous collagen (2 mg/ml) in the short-term culture. Decrease of MT1-MMP expression in BAPN treated culture may implicate the role of maturation of collagen for the transcriptional regulation without disturbing the activation level of MMP-2, which may be maintained by the non-transcriptional regulation of MT1-MMP. Again, however, the amount of collagen secreted by BAPN-treated cells was lower.

Mov13-5 CM mouse fibroblasts were introduced into the endogenous response model as another means to test the requirement of collagen fibril formation in the activation of MMP-2. The collagen produced by these cells lacks the ability to form fibrils since the cleavage site for removal of the C-propeptide domain has been mutated. Surprisingly, the pC-procollagen secreted by these cells was quite effective in stimulating MMP-2 activation either in the ascorbate-treated fibroblast culture or when transferred exogenously to normal cells. The lack of fibril formation was confirmed by SDS-PAGE analysis, suggesting that this collagen can adopt a permissive configuration without forming the actual fibril. Prolyl 4-hydroxylase inhibition was used to confirm the collagen specificity of the MMP-2 activation in these cells.

This result contrasts that with the specific peptide inhibitor of fibril formation and exogenous collagen. Due to budgetary constraints, we were unable to use the peptide with the pC-procollagen harvested from those cultures to attempt to block whatever

structure they do make. It is possible that the peptide would inhibit formation of whatever structure the Mov13-5 CM procollagen makes after cleavage by procollagen N-proteinase from either normal skin fibroblasts or Mov13-5 CM. Some evidence exists that pC-procollagen is able to form a fibril-like structure in the test tube (Suzuki et al., 1996), however in other studies, the removal of the C-propeptide domain was prerequisite to fibril formation *in vitro* (Kadler et al., 1987). It is worth noting that in the previous and present studies, evidence of the non-specific cleavage of the C-propeptide domain by a serine proteinase has been shown in cultures (Bateman et al., 1987). This is consistent with the just-detectable amount of collagen deposition in the long term-culture of Mov13-5 CM, and could explain the disparity between *in vitro* and *in vivo* observations.

The precise biochemical mechanism of collagen fibril assembly and growth *in vivo* is less well defined than the current understanding, which based largely on *in vitro* experiments (Payne et al., 1986). However fibril formation by pN-procollagen does occur, and the diseases resulting from this collagen malformation, type VIIC Ehlers-Danlos syndrome in humans and dematosparaxis in cows and sheep, are well documented (Nusgens et al., 1992; Smith et al., 1992). However, in the short term-culture performed here, the proportion of the completely processed collagen to total secreted collagen was low, and probably insufficient to serve as the major inducer of MMP-2 activation in these cultures. Therefore the majority of MMP-2 activation was due to the C-procollagen, presumably in a soluble fibril-like structure.

Ascorbate-Treated Fibroblast Culture Model

The ascorbate-treated fibroblast culture model is more complete than the addition of exogenous collagen, in the sense that the condition better resembles the situation that occurs *in vivo*, rather than the artificial system of exogenous collagen addition. The *in vivo* studies of *in situ* synthesis of collagen fibers in chick embryonic tendon demonstrated the possibility that secreted collagen could align normally as the 4-D stagger to 1-D stagger, or alternatively, could form the structure called segment-long-spacing (SLS) in which collagen molecules align in parallel without any stagger. These kinds of structures occurred in the narrow long spaces between the cytoplasmic processes of the cells, and appeared to form during the collagen secretion. These narrow long processes of the cells formed from the elongated vacuoles containing collagen molecules, fuse in tandem such that the end of the compartment becomes open to the extracellular space. Within the compartment, collagen molecules appeared to align together and assemble to form fibril segments approximately 10μm long, suggesting that procollagen is probably secreted in an aggregated form, and not as individual molecules (Bruns et al., 1979; Trelstad and Hayashi, 1979; Hulmes et al., 1983; Birk et al., 1990). However, soluble collagen was detected in the culture medium within one day of ascobate treatment, and found only by day 2 in the matrix by silver stain. These observations suggest that procollagen is secreted before precipitation on the monolayer, and further processed to fibrils. There have not been any studies that showed exactly where fibrils first formed in the culture system. Furthermore, these observations have been reported in normal cells, and the deposition processes may be different in transformed cells or with mutant collagens. It would be interesting to examine the cytoplasmic processes of the cells where

collagen is being secreted by electron microscopy. Mov13-5 CM and other cells, which we have used in the ascobate-treated fibroblast culture, may adopt fibril-like structures during the secretion process, and align their collagen to the extracellular compartment. This may explain the significant level of MMP-2 activation, which occurred in short-term, ascobate-treated Mov13-5 CM fibroblast cultures.

If the collagen is harvested and purified from the Mov13-5 CM culture media, and concentrated, it is capable of inducing activation. Presumably, part of the collagen in the 96-hour, ascorbate-treated culture is already associated with the cell layer and/or ECM, and is not transferred. However, this combination of exogenous and endogenous models is useful, and can be applied for both this and other fields of study, for example studies on the effects of each processed form of collagen using potent specific inhibitors or other strategies to disturb collagen processing.

MT1-MMP in MMP-2 Activation in Fibroblasts

In most of the previous studies, MT1-MMP has emerged as the prominent activator of MMP-2 rather than other MT-MMPs. Part of this may be because other MT-MMPs have not been explored as much as MT1-MMP. Similarly, the role of MT1-MMP as the activator of MMP-2 has exclusively been looked at in this study. Expression of MT1-MMP mRNA and protein was shown to be up-regulated by either exogenous or endogenous collagen, and was relative to the amount of collagen present. With the long-term ascorbate-treated cultures, MT1-MMP protein was up-regulated via the non-transcriptional component early in the culture period, and the transcriptional regulation of MT1-MMP mRNA was up-regulated later. It is not possible to determine whether more collagen is required for MT1-MMP

transcriptional regulation, or whether some important collagen maturation is required. The lack of MT1-MMP mRNA up-regulation seen with BAPN-treated endogenous collagen supports a requirement for collagen maturation, however BAPN also caused reduced collagen production.

Although we did not monitor other MT-MMPs, the role of MT1-MMP in mediating MMP-2 activation in response to exogenous and endogenous collagen was confirmed in this study with MT1-MMP knockout fibroblasts, as the level of activation achieved was relative to the ability of the cell to express MT1-MMP. However, in the ascorbate-treated fibroblast culture, non-MT1-MMP mediated MMP-2 activation was quite striking in MT1-MMP knockout cells. A panel of known proteinase inhibitors, including a potent inhibitor of matrix metalloproteinases, was used to test various possible proteinase families that could cause this activation, however none could abrogate or even decrease it. One possibility is that the enzyme causing this MMP-2 activation is rather functioning inside the cells, unable to be reached by the inhibitor(s). Preliminary Western analysis showed that active MMP-2 was present in the cytoplasmic component of cells in ascorbate-treated fibroblast culture. This is consistent with a previous report of intracellular MMP-2 activation in normal fibroblasts, apparently thourough a metalloproteinase-dependent process (Lee et al., 1997). Since expression levels of other MT-MMPs may have never been examined in these cells, it is possible that other MT-MMPs may mediate intracellular activation MMP-2 in longer-term, 3-dimensional fibroblast cultures. Although outside the scope of this study, it will ultimately be interesting to look further at what causes this activation, and where it takes place, using immunocytochemistry or cell fractionation techniques.

Concluding Remarks

In conclusion, collagen has emerged as a molecule of major interest in the area of MMP-2 activation, since it is physiologically available, and has a pronounced and specific ability to induce MMP-2 activation. More interestingly, in pathologic conditions such as cancer, newly synthesized collagen is detected, and can be related to the degree of disease progression. Many studies have investigated the mechanisms by which collagen regulates the MMP-2 activation process, but most have looked at the cell-receptor and signal transduction levels (Ellerbroek et al., 1999), and not many have focused on the structure of collagen (Azzam and Thompson, 1992). In this present *in vitro* study, we developed cell culture systems to refine our understanding of the apparent requirement of fibrillar collagen structure. Even though the two different culture systems developed here did not provide definitive explanations for this requirement, we learnt that only trace amounts of collagen can be sufficient for the induction of MMP-2 activation, and the ascorbate-treated fibroblast cultures gave better resolution of the non-transcriptional and transcriptional regulation of MT1-MMP by collagen. It is important to appreciate that there are limitations in interpretation of the ascorbate-treated fibroblast culture. This includes complex biological processes, which modulate the extent of the collagen propeptide domain processing, the ratio of co-assembled collagen types, and the presence of other matrix molecules and associated factors.

The very surprising finding in this system was the non-MT1-MMP-mediated component of MMP-2 activation. To the best of our knowledge, it appears either to involve unusual proteinases, or occur intracellularly, or in other cell compartments, which are protected from access by the inhibitors we used. This is entirely different to

what we see in the more commonly employed, exogenous model, and may represent an important *in vivo* pathway. Collectively, this context better approximates an *in vivo* model and may clarify and disclose the molecular and structural requirements of the real physiological environment.

Future Studies

Although outside the scope of this thesis, a number of new directions arise which could be pursued.

1. Functional consequences of periodate treatment on other collagen responses (eg. integrin binding efficiency) which may lead to the further study of auxiliary, carbohydrate-dependent, non-integrin glycoprotein surface receptors.
2. Electron microscopic analysis of the structure of collagen occurring in long-term culture Mov13-5 CM fibroblasts.
3. Clarification of the putative intracellular activation of MMP-2 in MT1-MMP-/- fibroblasts.
4. Investigation of the specific domains of MT1-MMP required for collagen-induced MMP-2 activation, using transfection models with mutated MT1-MMP and chimeras.