Matrix Metalloproteinase Expression in Normal Skin Associated With Basal Cell Carcinoma and in Distal Skin From the Same Patients

Nathan Monhian, MD; Brian S. Jewett, MD; Shan R. Baker, MD; James Varani, PhD

Objective: To obviate the difficulty of ruling out confounding variables (eg, age, individual variability) as the source of differences seen when comparing tumor tissue and control tissue from unrelated individuals, we examined the expression of matrix metalloproteinase (MMP)-1 (interstitial collagenase) and MMP-9 (92-kDa gelatinase B) in histologically normal skin immediately adjacent to basal cell carcinomas (peritumoral tissue) after Mohs micrographic surgery and postauricular skin from the same patients.

Design: Peritumoral and postauricular skin samples were obtained from 17 patients undergoing Mohs surgery. Expression of MMP-1 and MMP-9 was examined in these specimens using a combination of approaches including zymography, collagen-degradation assays, and immunohistology.

Results: The expression levels of MMP-1 and MMP-9 were consistently elevated in the peritumoral tissue compared with skin from the distal site.

Conclusion: This finding indicates that even when potentially important variables such as age and individual variability are controlled for, tumor-specific effects on the expression of MMP-9 and MMP-1 remain.

Arch Facial Plast Surg. 2005;7:238-243

THE MATRIX METALLOPROTEINASES (MMPs) are a family of genetically related enzymes that play a role in normal tissue development, remodeling, and repair. These same enzymes are also responsible for tissue destruction in several pathological conditions including acute and chronic inflammation, for skin damage occurring as a consequence of the natural aging process and following acute UV light exposure, and for connective tissue destruction during tumor invasion. In regard to the process of tumor invasion, MMPs are thought to play many important roles that promote tumor spread. Among these are destruction of the surrounding stroma to facilitate tumor cell penetration and localized destruction of basement membrane around endothelial cells as part of the angiogenic response. Basal cell carcinoma is the most common form of malignant skin neoplasm, accounting for 77% of all cases. Sun exposure is the major risk factor for this tumor, with 90% of the cases occurring on the head and neck. Basal cell carcinoma rarely metastasizes but is locally invasive and highly destructive. Concentrations of matrix metalloproteinases with collagenolytic activity as well as enzymes with gelatinolytic activity have been shown to be elevated in basal cell carcinoma tissue relative to normal skin. While the tumor cells, themselves, are capable of elaborating MMPs, most of the tissue-destructive enzymes detected in tumor tissue are thought to reflect production by the associated stroma. Because these same enzymes are also stimulated in response to acute UV irradiation, it is possible that enzyme up-regulation reflects (at least to some extent) recent sun exposure rather than the influence of the tumor per se. Likewise, MMP levels increase as a consequence of the natural aging process, and when comparisons are made between tumor-associated skin and normal skin from different individuals, inherent individual variability as well as age differences may contribute to altered levels of expression. In the present study we examined MMP expression in histologically normal skin immediately adjacent to the tumor in 17 cases of basal cell carcinoma after Mohs micrographic surgery.
along with postauricular skin from the same patients as control. We report herein that even when differences in age and individual variability are controlled for, levels of 2 MMPs—MMP-1 (interstitial collagenase) and MMP-9, (92-kDa gelatinase B)—are still consistently elevated in peritumoral tissue compared with postauricular skin.

METHODS

TISSUE

A normal skin specimen immediately adjacent to basal cell carcinoma tissue (peritumoral tissue) was obtained after Mohs micrographic surgery from 17 subjects seen at the Head and Neck Surgery Unit, Department of Otolaryngology, University of Michigan Hospitals, Ann Arbor. The basal cell tumors were all from the scalp, face, or nose, and the adjacent tissue was obtained from immediately beyond the margin of the tumor. Each peritumoral tissue sample was matched to a postauricular skin biopsy specimen obtained from the same patient as control. All procedures involving human subjects were approved by the University of Michigan institutional review board, and all subjects provided written informed consent prior to inclusion in the study.

MMP IMMUNOHISTOLOGICAL ANALYSIS

Tissue fixed in OCT (optimal cutting temperature) immediately after the biopsy was examined by immunoperoxidase staining for MMP-1 and MMP-9 expression as described previously. A rabbit polyclonal IgG antibody to MMP-1 was obtained from Chemicon, Temecula, Calif. Mouse monoclonal antibody to MMP-9 was obtained from Oncogene Sciences, Cambridge, Mass. Mouse monoclonal IgG1 (Calbiochem, San Diego, Calif) and a rabbit polyclonal IgG (R & D Systems, Minneapolis, Minn) were used as controls.

ORGAN CULTURE

Immediately after obtaining a biopsy specimen, the tissue was immersed in culture medium consisting of keratinocyte basal medium (KBM) (Cambrex, Walkersville, Md) and transported to the laboratory. Keratinocyte basal medium is a low-Ca\(^{2+}\) (0.15mM) serum-free modification of serum-free MCDB-153 medium (Sigma-Aldrich, St Louis, Mo) optimized for high-density keratinocyte growth.\(^6\) The culture medium was supplemented with calcium chloride to bring the final Ca\(^{2+}\) concentration to 1.4mM. This was done because previous studies have shown that survival of human skin in organ culture depends on a Ca\(^{2+}\) concentration that is optimized for fibroblast survival and growth.\(^1\) On arrival in the laboratory, the biopsy specimens were cut in pieces approximately 2 mm on a side and incubated in wells of a 24-well dish containing 0.5 mL of Ca\(^{2+}\)-supplemented KBM (5-6 tissue pieces per well). Cultures were incubated at 37°C in an atmosphere of 95% air and 5% carbon dioxide. Incubation was for 3 days. At the end of the incubation period, the culture medium was collected and assessed for expression of MMP-1 and MMP-9. After the 48-hour incubation period, the flasks were exposed to a mixture of trypsin and EDTA to release cells from the monolayer. Although both keratinocytes and fibroblasts were released with this treatment, only fibroblasts subsequently re-attached. Thus, we were able to isolate pure populations of fibroblasts from the 2 skin sites. Culture fluids were obtained from fibroblasts and handled exactly as the original mixed culture fluid.

SUBSTRATE-EMBEDDED ZYMOGRAPHY

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis substrate-embedded zymography (zymography) was used to identify enzymes with collagenase and gelatinase activities. Assays were carried out as described in a previous article.\(^6\) Zymographic images were converted to negative images and digitized. Quantification was accomplished by determining the number of pixels in the negative images. For organ cultures, data were normalized per biopsy specimen. For cell cultures, data were normalized to equivalent cell numbers. Gelatin zymography is useful for detection of MMP-2 (72-kDa gelatinase A) and MMP-9. β-Casein zymography is useful for detection of MMP-1, which appears as a doublet in the 54-kDa region of the gel.

COLLAGEN DEGRADATION ASSAY

Acid-solubilized (rat tail) fibrillar type I collagen was obtained from Becton-Dickinson, Bedford, Mass. Native collagen was diluted to 1 mg/mL in a phosphate-buffered saline solution (pH 7.2) and incubated at 37°C for 18 hours with peritumoral and postauricular organ culture fluids. At the end of the 18-hour incubation period, samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The presence of active enzyme was determined, based on the disappearance of the parental α\(_1\)- and α\(_2\)-collagen bands and the appearance of lower molecular weight fragments.\(^6\)

COMPARISON OF PERITUMOR AND POSTAURICULAR TISSUE FOR MMP-1 AND MMP-9 EXPRESSION

In the first series of experiments, 17 matched pairs of peritumoral and postauricular tissue were maintained in organ culture for 72 hours. Culture fluids collected at the end of the incubation period were assessed for the presence of MMP-9 by gelatin zymography and for MMP-1 by β-casein zymography (Figure 1). It can be seen from the quantitative data that MMP-9 levels were higher in peritumoral specimens than in the corresponding postauricular tissue. In addition to detecting MMP-9, gelatin zymography is also useful for detecting MMP-2. Consistent with past studies showing resistance of MMP-2...
to factors that stimulate MMP-9, virtually the same level of MMP-2 was observed in organ culture fluids from peritumoral and postauricular tissue. Quantitative data obtained from casein zymograms are also shown in Figure 1. It can be seen that MMP-1 levels were elevated in peritumoral specimens relative to postauricular tissue, but differences between the 2 tissue groups were not as striking as those seen with MMP-9.

Figure 2 shows representative gelatin and β-casein zymograms with samples from the 2 tissue sites. In addition to demonstrating quantitative differences in MMP-9 and MMP-1 expression between the 2 tissue sites, it can also be seen that latent and active enzyme forms were present in specimens from both sites. In comparing the entire group, there did not appear to be major differences between the 2 tissue sites in the relative levels of active vs latent enzyme forms.

Results from collagen digestion assays are shown in Figure 3. It can be seen from the quantitative data that exposure of intact collagen to culture fluids from peritumoral specimens resulted in a reduction of intact α1 and α2 chains of type I collagen ranging from approximately 30% to greater than 90%. In comparison, when type I collagen was exposed to culture fluid from postauricular tissue, reduction in α1 and α2 chains of type I collagen ranged from 10% to approximately 90%. On average, the percentage of degradation was higher with organ culture fluid from peritumoral specimens than from postauricular tissue (67% vs 43%, respectively), but this difference was not statistically significant (P > .05, t test).

In addition to assessing the loss of intact α1 and α2 chains of type I collagen and their conversion to three quarter- and one quarter–sized chains, we also identified the further conversion of these fragments to smaller peptides as a measure of gelatinolytic activity. In 8 of 10
specimens from postauricular tissue, the three quarter- and one quarter–sized fragments constituted the major fragmentation products. In contrast, the degradation pattern was much more complex with virtually all of the peritumoral specimens. The three quarter- and one quarter–sized fragments were decreased and replaced with multiple, smaller fragments (not shown). Thus, there was a good correlation between the presence of active gelatinolytic enzymes and extensive fragmentation of the collagen substrate.

**MMP-1 AND MMP-9 LOCALIZATION STUDIES**

Additional studies were carried out to identify cellular sources of MMP-1 and MMP-9 in peritumoral specimens. Two approaches were used for this. These included immunohistochemical staining of frozen tissue sections and enzyme measurements on cells grown in monolayer culture. Figure 4 shows results from immunohistology. Most of the staining for MMP-9 was associated with epidermal keratinocytes. Staining was seen in the basal layer of normal keratinocytes as well as in intradermal glandular structures. Staining of the dermal microvasculature was also apparent in some sections. Although there was variability in the extent and intensity of staining, the staining pattern shown herein was consistent among the entire group of specimens examined.

We also examined the tumor itself from several basal cell carcinomas. Staining for MMP-9 was seen in tumor cells from several specimens. Staining was variable from tumor to tumor. In contrast to MMP-9, which was detected mainly in epithelium and endothelium, MMP-1 staining was observed primarily in interstitial cells (presumed to be fibroblasts) (Figure 4).

To further define the cellular sources of MMP-9 and MMP-1, peritumoral and postauricular tissues were cultured in such a manner as to allow both epidermal keratinocytes and dermal fibroblasts to grow out from the tissue. Culture fluid (Ca²⁺-supplemented KBM) collected from these cultures after 48 hours of incubation was assayed by gelatin and β-casein zymography. High levels of both MMP-9 and MMP-1 were detected in the culture fluids. Active as well as latent forms of both enzymes were detected. There were no apparent differences between cells grown out from peritumoral vs postauricular tissue (Figure 5). Matrix metalloproteinase-2 was also detected in gelatin zymograms; again there was no major difference between tissue sources.

Finally, fibroblasts were isolated from the mixed cultures and passaged 1 to 2 times. Culture fluids (Ca²⁺-supplemented KBM) were collected from cultures of isolated fibroblasts and assayed by gelatin and β-casein zymography. High levels of MMP-1 were detected, but in contrast to what was observed in mixed culture, virtually all of the activity was in the latent form. No dif-

---

Figure 4. Immunohistological detection of matrix metalloproteinase (MMP)-9 (A and B) and MMP-1 (C and D) in peritumoral tissue. Matrix metalloproteinase-9 was detected in the normal epithelium of virtually every specimen. In the basal epithelium (A), occasional positive cells were observed (arrows). Strong staining was observed in intradermal glandular structures (B) (arrows). Matrix metalloproteinase-1 was detected in interstitial stromal cells (C) (arrows). In some sections (D), MMP-1 was entirely negative (hematoxylin-eosin, original magnification x160).
Basal cell tumors elaborate large amounts of several MMPs. Much of the MMP activity associated with these tumors originates in the stroma, although the tumor cells themselves and the adjacent normal epithelium also contribute. Although high-level MMP expression is seen in basal cell carcinoma, it is difficult to know for certain if enzyme levels observed in tumor and peritumoral tissue are significantly different from levels in nontumor-associated skin since appropriate control tissue from distal sites in the same individual is often unavailable for comparison. In the past, skin from healthy volunteers has been used for control, but our own studies have demonstrated that age-related differences in enzyme expression levels as well as individual variability can be large. As a way to obviate the methodological difficulties inherent with comparisons between tumor-associated and tumor-nonassociated tissue from different individuals, we have in the present study assessed MMP levels in normal skin adjacent to surgically extirpated basal cell carcinomas and distal (postauricular) skin in the same individuals taken as control. Our results demonstrated that even when age and individual variability are controlled for, expression of both MMP-1 and MMP-9 is still elevated in tumor-associated normal skin compared with expression levels in distal skin.

What accounts for enzyme elevation in tumor-associated tissue? Presumably, factors elaborated by tumor cells induce MMP-1 and MMP-9 elaboration in the adjacent normal tissue. The nature of the relevant factors has not been determined with certainty, although previous studies have identified candidate molecules. Agonists for the epidermal growth factor receptor constitute one potentially important category of molecules. Epidermal growth factor receptor ligand levels are elevated in many tumors, and epidermal growth factor receptor activation results in signaling events that lead to formation of the AP-1 transcription factor and MMP gene transcription. Endogenously generated ligands for epidermal growth factor receptor such as heparin-binding epidermal growth factor and amphiregulin could contribute to MMP induction via activation of mitogen-activated protein kinase (MAPK) signaling and AP-1 formation. It is unlikely, however, that such molecules are the only driving force. Epidermal growth factor receptor activation results in epidermal proliferation (leading to epidermal hyperplasia) as well as MMP induction. While hyperplasia of the normal epithelium adjacent to basal cell tumors occurs, it is not universal.

Interleukin 1 (IL-1) may also be an important MMP-inducing agonist in basal cell tumors. High levels of both IL-1α and IL-1β are present in skin, and IL-1 is a potent inducer of MMP gene transcription. Recent studies from our laboratory showed that induction of MMP-1 in dermal fibroblasts by culture fluids from epidermal keratinocytes could be largely inhibited in the presence of IL-1 receptor antagonist. Interleukin 1 stimulation was related to induction of p38 MAPK in dermal fibroblasts. While these past studies are suggestive, further work will be required to elucidate the exact roles of these and other factors as inducers of MMP expression in basal cell tumors.

Several additional issues need to be addressed. First, is the possible involvement of sun exposure in MMP up-regulation? Matrix metalloproteinase induction occurs as a consequence of acute UV exposure and it may be that some of the enhanced enzyme expression in tumor-associated skin reflects a response to solar radiation rather than an effect of the tumor itself. While postauricular skin, itself, is sun-exposed, whether it is as sun exposed as skin from the face is difficult to say. What can be said, however, is that while acute UV exposure stimulates MMP up-regulation, chronically sun-damaged skin (ie, skin that is damaged by years of exposure to the sun) is generally not different from the corresponding sun-protected skin in regard to MMP levels. Thus, even if postauricular and peritumoral skin were not equivalent in the overall level of sun damage, this would only be an issue in cases where the patient was exposed to excessive solar irradiation immediately prior to surgery (an unlikely scenario).

A second issue concerns the relevance of the enzyme findings in regard to actual connective tissue damage. Most MMPs are secreted as latent enzymes and activated extracellularly. The identification of active as well as latent forms of both MMP-1 and MMP-9 by zymography clearly indicates that activation has occurred. Even identification of active enzyme forms in zymography, however, does not prove that the enzymes were active in the tissue since MMP inhibitors are often secreted in conjunction with the enzymes. The collagen-degradation studies (Figure 3 and related text) address this issue. The same organ culture fluids that demonstrated active enzyme forms by zymography were capable of cleaving intact native collagen to smaller fragments. Thus, it can be assumed that whatever inhibitor levels were present in the organ culture fluids, there was enough active MMP-1 and MMP-9 to overcome this level of inhibitor. Of fur-
ther interest, while culture fluids from both peritumoral and postauricular skin produced the typical one quarter– and three quarters–sized peptides from intact collagen, culture fluid from peritumoral skin produced a more extensive degradation pattern—the initial one quarter– and three quarters–sized fragments were further cleaved to a variety of smaller fragments. Thus, to the extent that what occurs in organ-cultured tissue is a reflection of what occurs in intact tissue, the implication is that the peritumoral environment would contain a more extensively damaged matrix.

Another issue concerns the cells responsible for elevated levels of MMP-9 and MMP-1 expression in tumor-associated skin. Consistent with previous findings, our data strongly suggest that epithelial cells are primarily responsible for the MMP-9 expression detected in the organ culture fluids while MMP-1 expression is a product of stromal cells. This is based on immunohistochemical findings as well as results from studies with cells in culture. Findings from cell culture comparisons demonstrated no significant differences in production of either MMP based on tissue site. This could indicate that the factors responsible for MMP induction are short lived or quickly diluted out under culture conditions. Alternatively, the process of maintaining cells in monolayer culture may, itself, influence MMP production and, thereby, “mask” tumor-related influences.

In summary, previous work has suggested that MMPs are up-regulated in tumor tissue and participate in the invasion process. In the past, comparisons between tumor tissue and control tissue from unrelated individuals have often been used. The findings from this study show that even when individual variability is controlled for, elevated expression of MMP-9 and MMP-1 in skin associated with basal cell carcinomas remains.

Accepted for Publication: November 22, 2004.
Correspondence: James Varani, PhD, Department of Pathology, University of Michigan, 1301 Catherine Rd, Box 0602, Ann Arbor, MI 48109 (Varani@umich.edu).
Funding/Support: This study was supported in part by grant DK59169 from the US Public Health Service, Bethesda, Md.
Additional Information: While carrying out this research, Drs Monhijon and Jewett were fellows of the American Academy of Facial Plastic and Reconstructive Surgery under the mentorship of Dr Baker.

REFERENCES