Jpn. J. Cancer Res. 84, 409–418, April 1993

The Expression of Invasive Behavior of Differentiated Squamous Carcinoma Cell Line Evaluated by an *in vitro* Invasion Model

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In order to elucidate the factors contributory to the expression of invasiveness of oral squamous cell carcinoma, we conducted biochemical and morphological comparisons of well differentiated squamous carcinoma cell line OSC-19 (oral squamous cell carcinoma) and undifferentiated carcinoma cell line KB, both cultured on 3T3 cell-embedded collagen gel (*in vitro* invasion model). OSC-19 cells invaded 3T3 cell-embedded collagen gel, while KB cells and OSC-19 cells on 3T3 cell-free gel matrix were less invasive. Cultured OSC-19 cells were characterized by lower proliferating activity, lower secretion of laminin and higher secretion of fibronectin than those of KB cells. Although the basement membrane with deposition of laminin and type IV collagen was formed, it was discontinuous at the invasion front. Gelatin zymography and western blotting showed matrix metalloproteinases (MMP), i.e., 72 kDa gelatinase (MMP-2) and 92 kDa gelatinase (MMP-9). Gelatinolytic activity was assayed, and was higher in OSC-19 cells than in KB cells or OSC-19 cells of the 3T3 cell-free model. By immunohistochemical analysis, MMP-2-positive cells were found scattered in both cell lines without any preferential localization, and the positivity for MMP-9 was localized in the invasion front of OSC-19 cells. These results strongly suggest that the invasiveness of squamous cell carcinoma is well correlated with cell-matrix adhesion by fibronectin and with focal elaboration of metalloproteinases, especially MMP-9, which play a major role in degrading the extracellular matrix components.

Key words: Oral squamous cell carcinoma — Invasion model — Fibronectin — Basement membrane — Matrix metalloproteinase

The invasive ability of carcinoma cells is one of the most important characteristics of the malignant tumor. The invasiveness seems to be attributed to multiple factors such as cell growth, adhesiveness, enzyme production and motility, some of which may be influenced by cell communication with stromal cells, defense mechanisms of immunocompetent cells, vascular supply and the other physical and anatomical conditions. In order to understand better the complex mechanisms of tumor cell invasion, many *in vitro* invasion models have been developed and used with particular attention to invasive activity of malignant tumor cells into various biomatrices such as chick chorioallantoic membrane,[1,2] collagen gel,[3] reconstituted basement membrane[4,5] and fibroblast-embedded collagen gel.[6] Among them, the invasion model using fibroblast-embedded collagen gel appears to be useful, since the epithelial-stromal interaction modulates the invasive capacity of carcinoma cells,[6–10] and fibroblasts affect basement membrane formation and junctional activity of epithelial cells and epithelium-derived carcinoma cells.[11,12]

In the present study, we devised an *in vitro* invasion model with 3T3 cell-embedded collagen gel as substrata, and employed it to investigate the factors related to the invasive ability of squamous cell carcinoma into the gel matrix, in comparison with another type of carcinoma cell line. We present results indicating that higher production of fibronectin and elevation of certain enzymatic activities, particularly of 92 kDa gelatinase (MMP-9), are closely correlated with invasiveness of squamous carcinoma cells.

MATERIALS AND METHODS

Cell lines Cell lines derived from oral squamous cell carcinoma were used: KB cells[13] were obtained from the Japanese Cancer Research Resources Bank (JCRB). OSC-19 cells[14,15] were kindly gifted by Dr. Yokoi. Swiss 3T3 cells derived from mouse fetal fibroblasts were obtained from JCRB. The characteristics of the carcinoma cells were investigated by implantation in the subcutaneous tissue of nude mice (Balb/c/*nu/nu*).

Invasion model Three-dimensional type I collagen gel matrix composed of acid-soluble bovine collagen solution (pH 3.0, Nitta Gelatin, Tokyo), Eagle's minimal essential medium (MEM) and sodium bicarbonate was prepared in a 35 mm plastic dish.[16] The components were mixed with 3T3 cells (3×10^6 cells) at 4°C, and the gel matrix (2 ml) was formed by incubation at 37°C for 30

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min and cultured in MEM supplemented with 10% fetal calf serum (FCS) overnight in a CO_2 incubator. Next day, carcinoma cells (2×10^6 cells) were overlaid on the 3T3 cell-embedded collagen gel matrix. After the carcinoma cells had adhered to the underlying collagen gel, the gel was floated in the medium with a spatula. The investigation was conducted on days 4, 8, 14 and 21 in culture. To analyze the role of 3T3 cells, 3T3-cell-free substratum was used.

Histological investigation Both tumor masses grown in nude mice and cell-matrix specimens of the invasion model were fixed with 4% paraformaldehyde for light microscopy, dehydrated with an ethanol series, and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin. For electron microscopy, they were fixed with 2.5% glutaraldehyde and 2% osmium tetroxide, dehydrated with an ethanol series, and embedded in Epok 812. Ultrathin sections were stained with uranyl acetate and lead citrate.

Immunohistochemistry Paraffin sections were immunostained for laminin and type IV collagen by the streptavidin-biotin-peroxidase complex method.[17] Deparaffinized sections were pretreated with 0.4% pepsin (Sigma, MO, USA) in 0.01 *N* HCl for 20 min. Rat anti-human laminin (×2,000, Immunotech, Marseille, France) and mouse anti-human type IV collagen (×1,000, Shiseido, Tokyo) monoclonal antibodies were used as primary antibodies. Biotinylated rabbit anti-rat and -mouse IgG antibodies (×200, Vector Laboratories, CA, USA) were reacted and then streptavidin-biotin-peroxidase complex (Jackson Immunoresearch Laboratories, PA, USA) was applied. Visualization was done with H_2O_2-added diaminobenzidine tetrahydrochloride.

To immunolocalize matrix metalloproteinases (MMPs),monoclonal antibodies against human MMP-1 (tissue collagenase), MMP-2 (72 kDa gelatinase/type IV collagenase) or MMP-9 (92 kDa gelatinase/type IV collagenase) were used. These antibodies were prepared by using synthetic peptides corresponding to the amino acid sequences of the COOH-terminal domains of the MMPs (residues 332–351, VQGQNVLHGYPKDIYSSFG for MMP-1; residues 524–539, VTPRDKPMGPLLVATF for MMP-2; and residues 626–644, RSAEVDRMFPG-VPLDTHD for MMP-9) according to the method of Kodama *et al.*[18] Monospecificity of the antibodies and their applicability for immunostaining were proven by immunoblot analyses and immunostaining of the rheumatoid synovium (Okada *et al.*; manuscript in preparation). The specimens were treated with monensin as previously described,[19] and fixed with 4% paraformaldehyde. Paraffin sections were immunostained with streptavidin-biotin-peroxidase complex. Concentrations of the antibodies against MMP-1, -2 and -9 were 1.7, 3.5 and 2.0 μg/ml, respectively.

Measurement of extracellular matrix components in culture media Amounts of laminin, type IV collagen and fibronectin secreted into the culture media by carcinoma cells were measured by sandwich enzyme-linked immunosorbent assays (ELISA). The conditioned media were collected after cultivation for 2 days in SFM-101 medium (Nissui, Tokyo) supplemented with 0.2% lactalbumin hydrolysate and 0.1 m*M* ascorbic acid.[20] Anti-human laminin (×3,000, Calbiochem Corp., CA, USA), type IV collagen (×1,000, Shiseido) or fibronectin (×3,000, Calbiochem Corp.) monoclonal antibodies were adsorbed on wells of a microtiter plate. Bovine serum albumin was adsorbed for blocking nonspecific reaction. Purified human laminin, type IV collagen[21] and fibronectin[22] and the conditioned culture media were diluted serially and incubated in the wells. Then, anti-human laminin (×500, E-Y Laboratories, CA, USA), fibronectin (×1,000, DAKO, Glosrup, Denmark) or type IV collagen (×500, Advance, Tokyo) polyclonal antibodies were reacted. After application of alkaline phosphatase-conjugated anti-rabbit IgG antibodies (×500, Cappel, PA, USA), alkaline phosphatase substrate (Sigma) was incubated, and then the optical density at 405 nm was measured. In our assay system for fibronectin no cross-reaction with murine or bovine fibronectin was observed.

Gelatin zymography According to the method described by Hibbs *et al.*,[23] zymography using sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 0.2% gelatin (8% total acrylamide) was performed. The supernatant of the conditioned medium was electrophoresed under non-reducing conditions at 4°C. The gels were washed with 2.5% Triton X-100 for an hour, incubated in 0.05 *M* Tris-HCl buffer (pH 7.5) containing 0.15 *M* NaCl, 0.01 *M* $CaCl_2$ and 0.02% NaN_3 for 12 h at 37°C, and stained with Coomassie brilliant blue R250.

Assays for gelatin- and collagen-degrading activities Gelatinolytic and collagenolytic activities in the culture media were measured according to the methods described by Harris and Krane[24] and Cawston and Bar-rett,[25] respectively. The assays were performed at 37°C for 12 h in the presence of 1.0 m*M* *p*-aminophenylmercuric acetate for gelatinolytic activity and 1.5 m*M* *p*-aminophenylmercuric acetate for collagenolytic activity to activate the precursors of MMPs.[26] After centrifugation, radioactivities in the supernatants were measured with a scintillation counter. One unit of gelatinolytic and collagenolytic activities degrades 1 μg of [^{14}C]gelatin and [^{14}C]collagen per minute at 37°C, respectively.

Immunoblotting Proteins in the conditioned medium were precipitated with 33% (w/v) trichloroacetate and dissolved in the SDS sample buffer containing 2-mercaptoethanol. The samples were electrophoresed in SDS

polyacrylamide gel (8%), and transferred to a nitrocellulose membrane. The membrane was immunostained by the avidin-biotin-peroxidase method using anti-MMP-1, -2 and -9 antibodies.

Statistical analysis The significance of differences was analyzed by analysis of variance and Scheffé's test for variables using the statistics package of Statistical Analysis System (SAS).

RESULTS

Characteristics of OSC-19 and KB cells Doubling times of OSC-19 and KB cells cultured on plastic dishes were 57.8 and 26.7 h, respectively.

These tumor cells were completely different in cell shape, differentiation and infiltrating mode into the surrounding tissue of nude mice. OSC-19 cells were polygonal, and showed marked keratinization, forming a cancer pearl. They proliferated with a pavement-like arrangement and showed an invasive growth pattern with neutrophil infiltration and fibroblastic reaction in the peripheral zone (Fig. 1A). KB cells, on the other hand, had ovoid cell shape with scanty cytoplasm and there were many mitoses and prominent nucleoli. Keratinization and intercellular bridging were not observed. The KB cells grew in an expansive manner against the surrounding murine tissue with poor stromal reaction (Fig. 1B).

Electron microscopically, OSC-19 cells had abundant tonofilaments that often formed electron-dense bundles near the nuclei. They were connected to each other by rigid desmosomes and had numerous hemidesmosomes to which discontinuous basement membranes were attached. KB cells had poorly developed desmosomes and little intercellular connecting apparatus. The cytoplasm was endowed with abundant free ribosomes and a few intermediate filaments, but tonofilaments were scarcely seen (data not shown). Thus, KB cells used in the present experiment were considered to be epithelial but not squamous in nature.[27] We regarded KB cell line as an undifferentiated carcinoma and OSC-19 cell line as a well differentiated squamous cell carcinoma.

Morphological findings of OSC-19 and KB cells in an invasion model OSC-19 cells cultured on 3T3 cell-free substrata formed two or three cell layers and rarely invaded the gel (Fig. 2A). OSC-19 cells cultured on 3T3 cell-embedded collagen gel formed multi-layers up to 10 layers thick, with distinct squamous differentiation, and invaded the collagen gel matrix starting on day 8. Underlying 3T3 cells were transformed from ovoid to fusiform or asteroid cells (Fig. 2B). Ultrastructurally, these OSC-19 cells had numerous desmosomes, hemidesmosomal structure and distinct tonofilaments, and they extended slender cytoplasmic processes into the gel. The number of cells invaded was increased by days 14 and 21. Basement membrane structure was partially developed on day 8, linear and frequently interrupted on day 14, and continuous on day 21 (Fig. 3). OSC-19 cells cultured without 3T3 cells did not form a basement membrane until day 21 (Table I).

KB cells proliferated in multi-layers on the collagen gel with or without 3T3 cells. They showed ovoid shape with scant cytoplasm. There were many exfoliated and necrotic cells on the superficial layer, probably because of their rapid growth and poor coherence. 3T3 cells in the collagen substratum remained round and slightly elon-

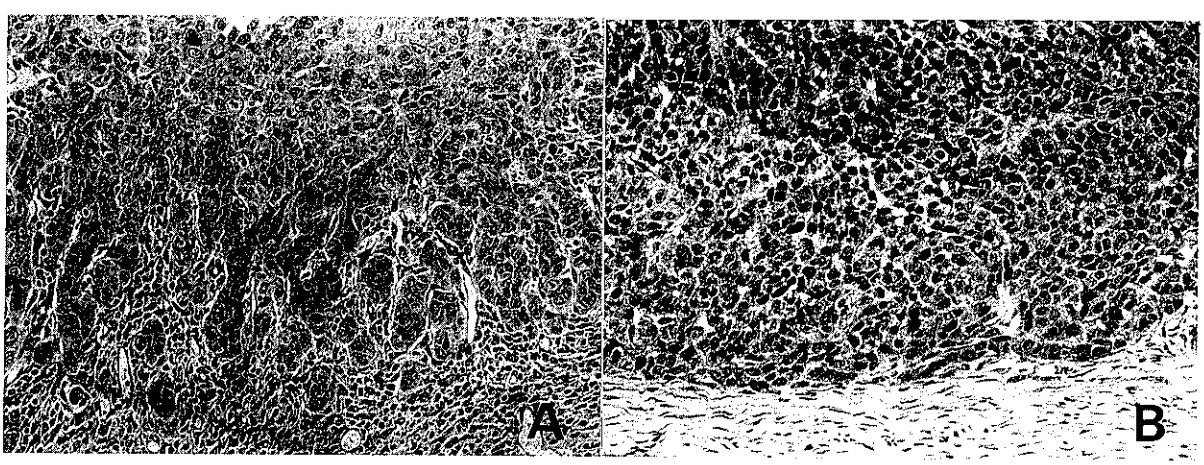


Fig. 1. Photomicrographs of carcinoma cells grown in the subcutaneous tissue of nude mice one week after injection. OSC-19 cells show invasive growth with fibroblastic reaction and inflammatory cell infiltration (A). KB cells show expansive growth without stromal reaction by fibroblasts (B). A, B ×240.

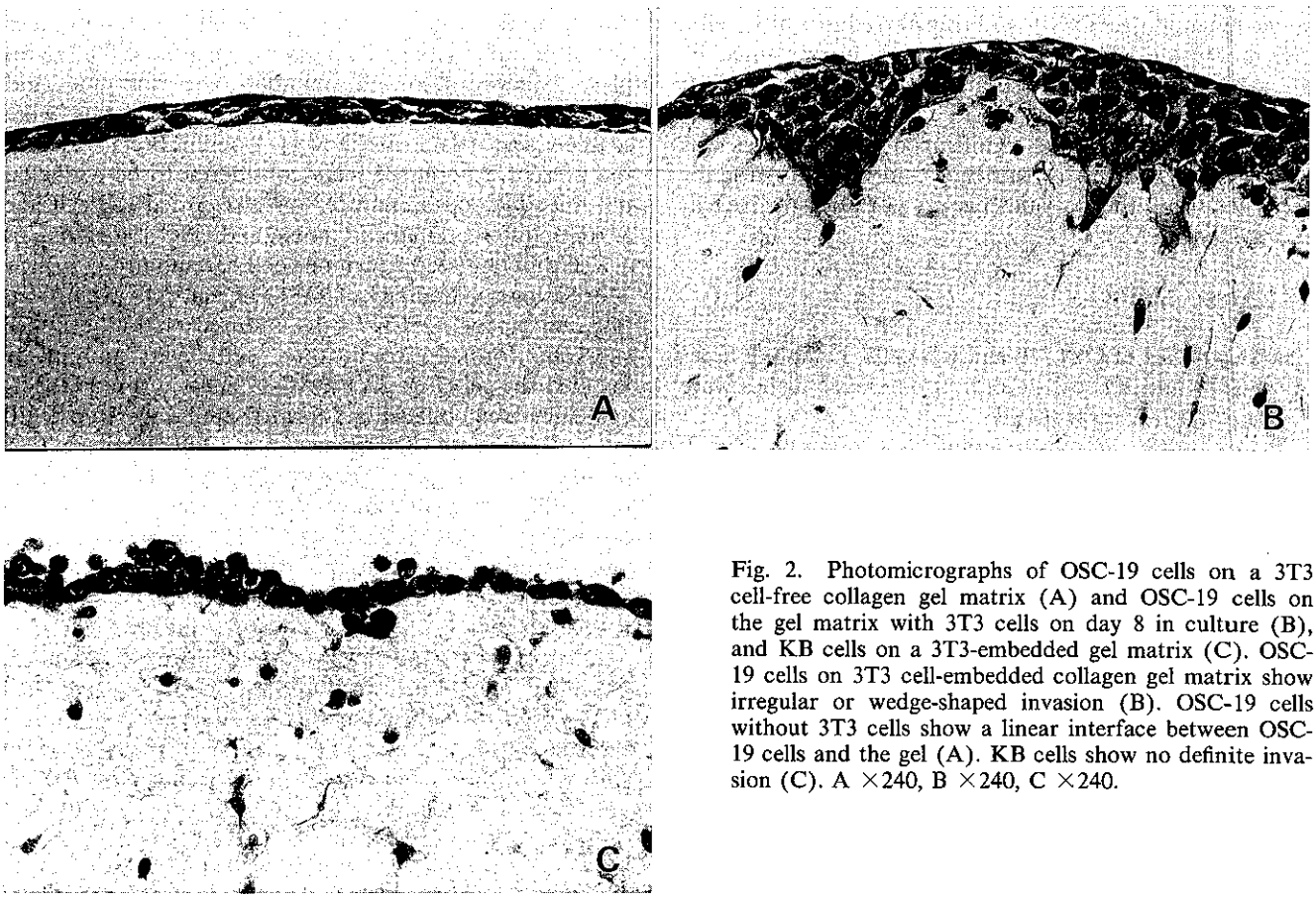


Fig. 2. Photomicrographs of OSC-19 cells on a 3T3 cell-free collagen gel matrix (A) and OSC-19 cells on the gel matrix with 3T3 cells on day 8 in culture (B), and KB cells on a 3T3-embedded gel matrix (C). OSC-19 cells on 3T3 cell-embedded collagen gel matrix show irregular or wedge-shaped invasion (B). OSC-19 cells without 3T3 cells show a linear interface between OSC-19 cells and the gel (A). KB cells show no definite invasion (C). A ×240, B ×240, C ×240.

gated in their cytoplasmic processes. KB cells scarcely penetrated into collagen gels (Fig. 2C). At the interface between KB cells and the collagen gel, no basement membrane structure was observed by electron microscopy (data not shown).

Deposition of extracellular matrix components secreted by carcinoma cells Laminin was immunolocalized on day 8 in a linear pattern only beneath the multi-layered OSC-19 cells cultured with 3T3 cells, initially around the invasion front (Fig. 4A). It was not immunostained in KB cells (Table I), although KB cells produced it constantly in the culture medium. Laminin released by OSC-19 cells increased with the advance of culture days and was influenced by 3T3 cells (Fig. 5).

Type IV collagen was immunolocalized on day 8 in culture in a linear pattern beneath the multi-layered OSC-19 cells with 3T3 cells (Fig. 4B) and KB cells with 3T3 cells (data not shown). 3T3 cell-free cultures did not show type IV collagen immunoreactivity in either OSC-19 or KB cells (Table I). In conditioned media type IV collagen was detectable only in KB cells with 3T3 cells,

Fig. 3. Electron micrograph of OSC-19 cells on day 21 in culture. Apparently continuous basement membrane (arrows) is formed. ×9,000.

but not in OSC-19 cells, although OSC-19 cells formed basement membranes. These results indicate type IV collagen produced by carcinoma cells was immediately deposited around the cells under the influence of 3T3 cells.

Table I. Summary of Immunohistochemical and Electron Microscopic Examinations on Basement Membrane Components and Basement Membrane Formation in an *in vitro* Invasion Model of Tumor Cells

	OSC-19 cells						KB cells					
	without 3T3			with 3T3			without 3T3			with 3T3		
Culture days	4	8	14	4	8	14	4	8	14	4	8	14
Immunostaining												
Laminin	−	−	−	−	+	+	−	−	−	−	−	−
Type IV collagen	−	−	−	−	+	+	−	−	−	−	+	+
Basement membrane by electron microscopy	−	−	−	−	−	+	−	−	−	−	−	−

Symbols: −, negative or absent; +, positive or present.

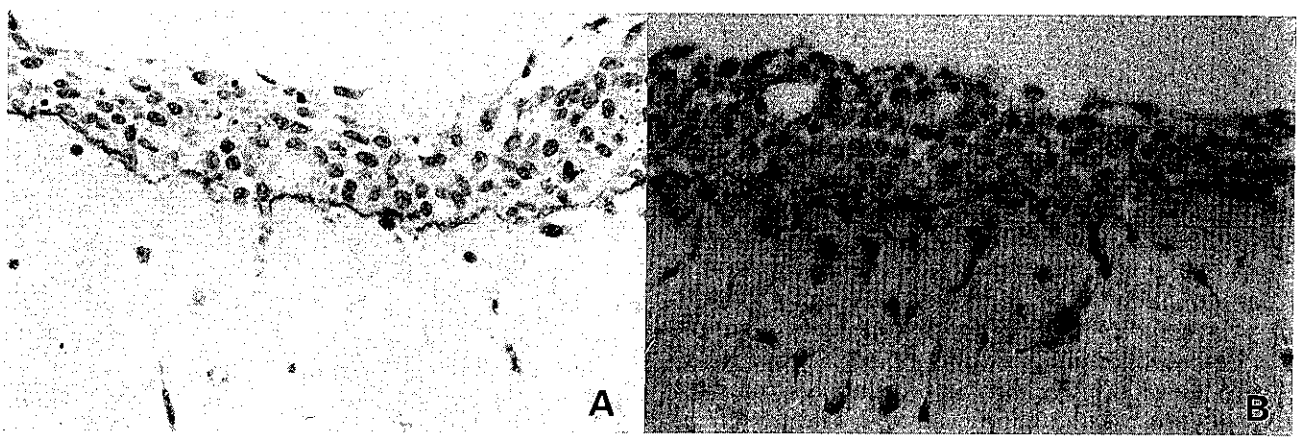
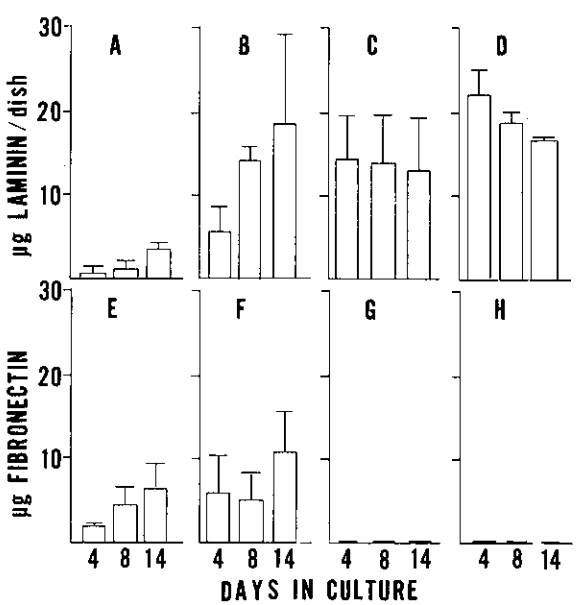


Fig. 4. Immunostaining for laminin (A) and type IV collagen (B) in OSC-19 cells on day 8 in culture. Note discontinuity of basement membrane components, laminin and type IV collagen, at the site of invasion. A, B ×140.



Fibronectin was detected in OSC-19 cell series, but not in KB cells. The amounts were higher in OSC-19 cells with 3T3 cells ($P<0.05$) (Fig. 5). Fibronectin was not immunostained in KB or OSC-19 cells, probably because the concentration of aggregates was below the limit of sensitivity of the antibody.

MMPs in conditioned culture media Gelatin zymography of the culture media from the invasion model showed several gelatinolytic activities with different molecular weights (Fig. 6). The digestion patterns of the samples from the 3T3 cell-free groups of KB and OSC-19 cells

Fig. 5. Content of laminin and fibronectin in OSC-19 cells without 3T3 cells (A, E), OSC-19 cells with 3T3 cells (B, F), KB cells without 3T3 cells (C, G) and KB cells with 3T3 cells (D, H) in conditioned culture media. Mean and standard deviation of triplicate determinations.

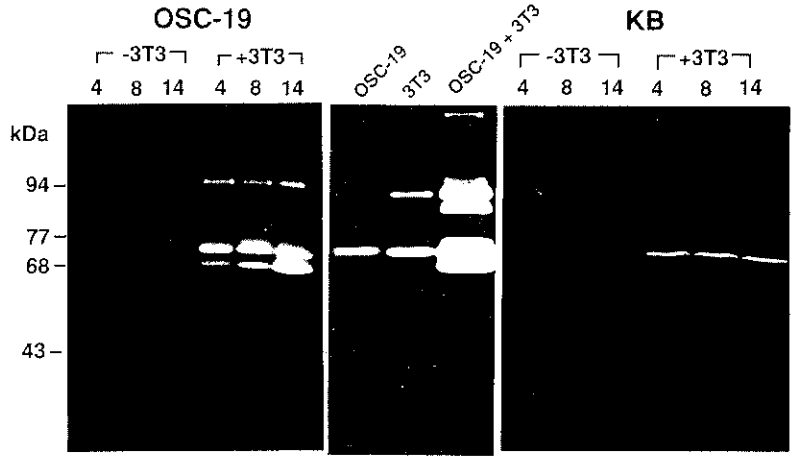


Fig. 6. Zymograms using gelatin substrate-acrylamide gel (8%) electrophoresis of conditioned culture media of OSC-19 and KB cells with or without 3T3 cells in collagen gels. In order to identify the products of 3T3 cells and OSC-19 cells, conditioned culture media of OSC-19 cells cultured on the 3T3-free collagen gel (OSC-19), 3T3 cells alone in the collagen gel (3T3) and OSC-19 cells cultured on the 3T3-embedded collagen gel (OSC-19+3T3) were run simultaneously, and the pattern is shown in the center of the figure. Numerals above (4, 8 and 14) indicate days in culture.

were almost the same: the major activity was associated with a band of 72 kDa. The same activity was also detected in the samples from both cell lines with 3T3 cells. Since the 72 kDa activity was observed in the media from either carcinoma cells or 3T3 cells alone cultured on a plastic dish, it was considered to represent the sum of enzymes secreted by carcinoma cells and 3T3 cells. The gelatinolytic activity of 95 kDa was detected in the 3T3 cell-embedded group of OSC-19, but not in the 3T3 cell-free group. This activity is probably derived from 3T3 cells because the same band was detected in the conditioned medium of 3T3 cells. Another gelatinolytic activity band with 92 kDa was faintly observed in all the conditioned media of the 3T3 cell-embedded groups.

Immunoblotting analyses revealed that all the samples from OSC-19 and KB cells contain MMP-2 (72 kDa gelatinase/type IV collagenase) and MMP-9 (92 kDa gelatinase/type IV collagenase) (Fig. 7).

In order to quantify these enzymatic activities, gelatinolytic and collagenolytic activities were measured using ^{14}C-labeled substrates. Gelatinolytic activity was significantly ($P<0.05$) higher in OSC-19 cells than in KB cells (Fig. 8). In addition, it was noted that levels of gelatinolytic activity in the samples from the 3T3 cell-embedded groups of KB and OSC-19 cells were significantly ($P<0.05$) higher than those of samples from the 3T3-free groups. No collagenolytic activity was detected in any of the samples from both cell lines.

Immunolocalization of MMPs OSC-19 and KB cells of the 3T3 cell-embedded groups were immunostained. MMP-9 was positively stained in a few OSC-19 cells, which were located in and around the wedge-shaped invasion front (Fig. 9A). KB cells were negative for MMP-9. MMP-2 was immunolocalized randomly in KB cells (Fig. 9B) and in OSC-19 cells, but 3T3 cells were not labeled. MMP-1 was never detected.

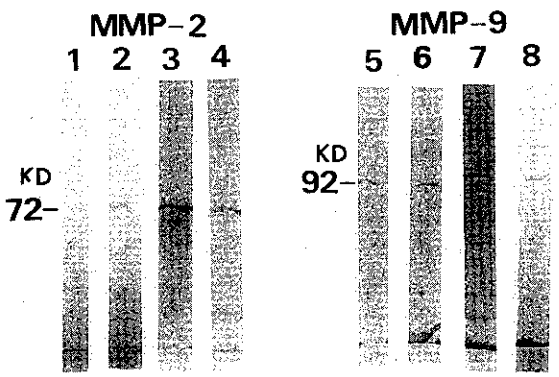


Fig. 7. Western blotting of conditioned media on day 8 using anti-MMP-2 and anti-MMP-9 for OSC-19 and KB cells. Lanes 1 and 5, OSC-19 without 3T3 cells; Lanes 2 and 6, OSC-19 cells with 3T3 cells; Lanes 3 and 7, KB cells without 3T3 cells; Lanes 4 and 8, KB cells with 3T3 cells.

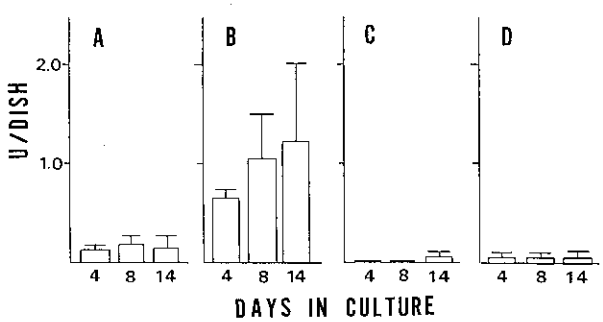


Fig. 8. Gelatinolytic activity in conditioned culture media. A, OSC-19 cells without 3T3 cells; B, OSC-19 cells with 3T3 cells; C, KB cells without 3T3 cells; D, KB cells with 3T3 cells. Mean and standard deviation of triplicate determinations.

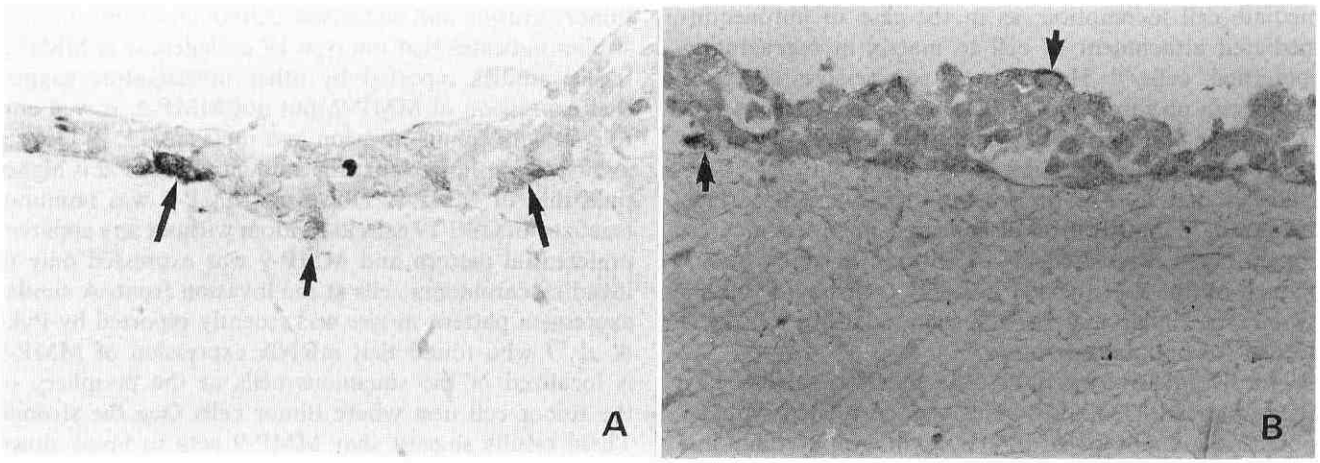


Fig. 9. Immunostaining for MMP-2 and MMP-9 on day 8. MMP-9 is positively stained in an OSC-19 cell at the invasion front (A, short arrow) and cells adjacent to it (A, long arrows). MMP-2 is randomly positive in KB cells (B). A ×240, B ×240.

DISCUSSION

In the present study, we employed 3T3 cells instead of human fibroblasts in an invasion model for evaluating the invasive capacity of tumor cells, because of their availability. The 3T3 cells appear to be a little different from fibroblasts in nature, even though they were derived from murine fetal fibroblasts, since we had to use 30 times as many 3T3 cells as compared with the human fibroblasts used in Matsumoto's model.[6] However, the present assay model is considered to be very useful in that it could provide us with reproducible data for analysis of factors related to invasiveness, especially with respect to the interaction of the carcinoma cells with stromal cells.

In the present invasion model, well differentiated squamous carcinoma cells (OSC-19) cultured with 3T3 cells definitely invaded the substrata of collagen gel, exhibiting basement membrane formation, positive immunoreactivity with anti-laminin and anti-type IV collagen antibodies, increased fibronectin production, increased amounts of metalloproteinases and gelatinolytic activity. It is surprising that OSC-19 cells formed basement membrane and deposits of type IV collagen and laminin simultaneously with invasion. Barsky *et al.*[28] reported that in benign epithelial neoplasm and carcinoma *in situ* the basement membrane was preserved, but it disappeared in an invasive carcinoma. Similarly, in poorly differentiated and invasive carcinoma of the colon and rectum[29, 30] endometrial carcinoma,[31] and pulmonary squamous cell carcinoma,[32] basement membranes are discontinuous, disrupted or absent. On the contrary, Gusterson *et al.*[33] reported that basement membranes did not disappear even in invasive squamous cell carcinomas, and proposed that the presence of basement membrane was not correlated directly with the invasion of carcinoma. Dingenans[32] found that both small cell clusters and singly invading cells are occasionally invested with continuous basement membranes, and assumed that such carcinoma cells are in a phase of temporary standstill of the invasive process. In our model, the basement membrane of OSC-19 cells seems to disappear at the invasion front, and concurrently basement membrane components, laminin and type IV collagen, were negative or discontinuously positive. These phenomena, therefore, seem to be common in the invasion process, particularly into gel matrix, by carcinoma cell lines of such a differentiated type.

Next, it is interesting that KB cells, which have high proliferative activity and are undifferentiated in nature, showed poor invasiveness, unlike OSC-19 cells. Thus, high proliferative activity and dedifferentiation did not appear to guarantee high invasive ability. Recently Nöel *et al.*[34] showed that highly malignant cell lines could not invade reconstituted basement membrane. Thus, invasive carcinoma cell phenotype may be correlated with other factors, including cell adhesiveness, cell motility, and matrix-degrading activity. Recently, adhesive proteins represented by laminin and fibronectin, and their cell surface receptors were shown to correlate closely with invasion and metastasis.[35] In the present model, in which fibronectin and laminin were assayed in conditioned media, it was noted that OSC-19 cells produced fibronectin and laminin and the amounts increased with development of invasion, while non-invasive KB cells constantly secreted laminin into the media during culture. Thus, invasive capacity into the collagen gel is possibly correlated with the adhering protein fibronectin, which acts to promote cell attachment to the gel matrix and to

mediate cell locomotion, as in the case of fibronectin-mediated attachment of cell to matrix in regenerating epidermal cells.[36] However, a suppressive effect of fibronectin on carcinoma cell invasion has been reported. D'Ardenne and Barnard[37] found that fibronectin usually disappeared at the invasion front, and Peltonen *et al.*[38] reported that fibronectin receptors on the cell surface membrane diminished or disappeared in invasive carcinoma. These reverse effects of fibronectin might be explained by the fact that an invading cell should lack its own extracellular matrix, but some adhesive matrix is needed for optimal migration.[39]

Thirdly, invasiveness of OSC-19 cells was enhanced by interaction with 3T3 cells and was correlated with an increased amount of MMPs by zymography. Our immunoblotting analyses have demonstrated that gelatinolytic activity bands of 72 kDa and 92 kDa corresponded to MMP-2 (72 kDa gelatinase/type IV collagenase) and MMP-9 (92 kDa gelatinase/type IV collagenase), respectively. On the other hand, 95 kDa gelatinolytic activity was also detected in the culture media of KB and OSC-19 cells with 3T3 cells, and that of 3T3 cells alone. MMP-9, which is a distinct gene product from MMP-2, is known to have slightly different molecular weights in the range of 92–97 kDa, depending on cell source and animal species.[40] Therefore, the gelatinolytic activity with 95 kDa is considered to be a product of murine 3T3 cells.

Liotta *et al.*[41, 42] have insisted that type IV collagen-degrading metalloproteinases play an important role in tumor invasion and metastasis. Although a series of their studies indicates that the type IV collagenase is MMP-2, recent studies reported by other investigators suggest that expression of MMP-9, but not MMP-2, is well correlated with tumor invasion and metastasis.[43, 44] Gelatin zymography showed that the amount of MMP-2 is higher than that of MMP-9. However, MMP-2 was immunolocalized in OSC-19 cells at random without any apparent preferential pattern and MMP-9 was expressed only in invading carcinoma cells at the invasion front. A similar expression pattern *in vivo* was recently reported by Pyke *et al.*,[45] who found that mRNA expression of MMP-9 is localized in the squamous cells at the periphery of the tumor cell nest where tumor cells face the stroma. These results suggest that MMP-9 acts to break down the stromal macromolecules at the local invading site, while MMP-2 may be related to extracellular matrix turnover. It would be of interest to know how OSC-19 cells incapable of producing MMP-1 degrade type I collagen during their invasion of the gel. Although previous studies indicated that MMP-9 can digest only type IV and V collagens and gelatin, as MMP-2 does, we have recently found that this proteinase can also cleave amino-terminal telopeptides of $\alpha 2$ chain of type I collagen.[46] It is, therefore, likely that type I collagen degraded by MMP-9 is denatured to gelatin and further digested into small fragments by the gelatinolytic activity. This cascade would occur at the invasion front in our model.

(Received October 14, 1992/Accepted January 18, 1993)

REFERENCES

1) Armstrong, P. B., Quigley, J. P. and Sidebottom, E. Transepithelial invasion and intramesenchymal infiltration of the chick embryo chorioallantois by tumor cell lines. *Cancer Res.*, **42**, 1826–1837 (1982).
2) Ossowski, L. In vivo invasion of modified chorioallantoic membrane by tumor cells: the role of cell surface-bound urokinase. *J. Cell Biol.*, **107**, 2437–2445 (1988).
3) Schor, S. L., Schor, A. M., Winn, B. and Rushton, G. The use of three-dimensional collagen gels for the study of tumor cell invasion *in vitro:* experimental parameters influencing cell migration into the gel matrix. *Int. J. Cancer*, **29**, 57–62 (1982).
4) Kramer, R. H., Bensch, K. G. and Wong, J. Invasion of reconstituted basement membrane matrix by metastatic human tumor cells. *Cancer Res.*, **46**, 1980–1986 (1986).
5) Albini, A., Iwamato, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M. and McEwan, R. N. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res.*, **47**, 3239–3245 (1987).
6) Matsumoto, K., Horikoshi, K., Rikimaru, K. and Enomoto, S. A study of an *in vitro* model for invasion of oral squamous cell carcinoma. *J. Oral Pathol. Med.*, **18**, 498–501 (1989).
7) Stoker, M., Gherardi, E., Perryman, M. and Gay, J. Scatter factor is a fibroblast-derived modulation of epithelial cell mobility. *Nature*, **327**, 239–242 (1987).
8) Stoker, M. and Perryman, M. An epithelial scatter factor released by embryo fibroblasts. *J. Cell Sci.*, **77**, 209–223 (1985).
9) Rosen, E., Goldberg, I. D., Liu, D., Setter, E., Donovan, M. A., Bhargava, M., Reiss, M. and Kacinsky, B. M. Tumor necrosis factor stimulates epithelial tumor cell motility. *Cancer Res.*, **51**, 5315–5321 (1991).
10) Picard, O., Rolland, Y. and Poupon, M. F. Fibroblast-dependent tumorigenicity of cell in nude mice. *Cancer Res.*, **46**, 3290–3294 (1986).
11) Mann, P. R. and Constable, H. Induction of basal lamina formation in epidermal cell cultures *in vitro*. *Br. J. Dermatol.*, **96**, 421–426 (1977).
12) Briggman, R. A. and Wheeler, C. E. Epidermal-dermal interactions in adult human skin: role of dermis in epidermal maintenance. *J. Invest. Dermatol.*, **51**, 454–465 (1968).
13) Eagle, H. Propagation in a fluid medium of a human

epidermoid carcinoma, strain KB. *Proc. Soc. Exp. Biol. Med.*, **89**, 362–364 (1955).
14) Yokoi, T., Yamaguchi, A., Odajima, T. and Furukawa, K. Establishment and characterization of a human cell line derived from a squamous cell carcinoma of the tongue. *Tumor Res.*, **23**, 43–57 (1988).
15) Yokoi, T., Homma, H. and Odajima, T. Establishment and characterization of OSC-19 cell line in serum- and protein-free culture. *Tumor Res.*, **24**, 1–17 (1988).
16) Wren, F. E., Schor, A. M., Schor, S. L. and Grant, M. E. Modulation of smooth muscle cell behavior by platelet-derived factors and the extracellular matrix. *J. Cell. Physiol.*, **127**, 297–302 (1986).
17) Garcia, R. L., Coltrera, M. D. and Gown, A. M. Analysis of proliferative grade using anti-PCNA/cyclin monoclonal antibodies in fixed, embedded tissues. Comparison with flow cytometric analysis. *Am. J. Pathol.*, **134**, 733–739 (1989).
18) Kodama, S., Kishi, J., Obata, K., Iwata, K. and Hayakawa, T. Monoclonal antibodies to bovine collagenase inhibitor. *Collagen Relat. Res.*, **7**, 341–350 (1987).
19) Okada, Y., Takeuchi, N., Tomita, K., Nakanishi, I. and Nagase, H. Immunolocalization of matrix metalloproteinase 3 (stromelysin) in rheumatoid synovioblasts (B cells): correlation with rheumatoid arthritis. *Ann. Rheum. Dis.*, **48**, 645–653 (1989).
20) Murad, S., Tajima, S., Jonson, G. R., Sivarajah, A. and Pinnell, S. R. Collagen synthesis in cultured human skin fibroblasts: effect of ascorbic acid and its analogs. *J. Invest. Dermatol.*, **81**, 158–162 (1983).
21) Ueda, Y. and Nakanishi, I. Immunohistochemical and biochemical studies on the collagenous proteins of human osteosarcomas. *Virchows Arch. B Cell Pathol.*, **58**, 79–88 (1989).
22) Kawahara, E., Shiroo, M., Nakanishi, I. and Migita, S. The role of fibronectin in the development of experimental amyloidosis. *Am. J. Pathol.*, **134**, 1305–1314 (1989).
23) Hibbs, M. S., Hasty, K. A., Seyer, J. M., Kang, A. H. and Mainardi, C. L. Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. *J. Biol. Chem.*, **260**, 2493–2500 (1985).
24) Harris, E. D. and Krane, S. M. An endopeptidase from rheumatoid synovial tissue culture. *Biochem. Biophys. Acta*, **258**, 566–576 (1972).
25) Cawston, T. E. and Barrett, A. J. A rapid and reproducible assay for collagenase using [1-^{14}C]acetylated collagen. *Anal. Biochem.*, **99**, 340–345 (1979).
26) Okada, Y., Tsuchiya, H., Shimizu, H., Tomita, K., Nakanishi, I., Sato, H., Seiki, K., Yamashita, K. and Hayakawa, T. Induction and stimulation of 92-kDa gelatinase/type IV collagenase production in osteosarcoma and fibrosarcoma cell lines by tumor necrosis factor α. *Biochem. Biophys. Res. Commun.*, **171**, 610–617 (1990).
27) Amano, F., Gottesman, M. M. and Pastan, I. Epidermal growth factor-dependent growth of human KB cells in a defined medium and altered growth factor requirements of KB mutants resistant to EGF-pseudomonus exotoxin conjugates. *J. Cell. Physiol.*, **135**, 502–508 (1988).
28) Barsky, S. H., Siegal, G. P., Jannotta, F. and Liotta, L. A. Loss of basement membrane components by invasive tumors but not by their benign counterparts. *Lab. Invest.*, **49**, 140–147 (1983).
29) Forster, S. J., Talbot, I. C. and Critchley, D. R. Laminin and fibronectin in rectal adenocarcinoma: relationship to tumor grade, stage and metastasis. *Br. J. Cancer*, **50**, 51–61 (1984).
30) Forster, S. J., Talbot, I. C., Clayton, D. G. and Critchley, D. R. Tumor basement membrane laminin in adenocarcinoma of rectum: an immunohistochemical study of biological and clinical significance. *Int. J. Cancer*, **37**, 813–817 (1986).
31) Stenback, F., Risteli, J., Risteli, L. and Wasenius, V. M. Basement membrane laminin and type IV collagen in endometrial adenocarcinoma: relation to differentiation and treatment. *Oncology*, **42**, 370–376 (1985).
32) Dingenans, K. P. What's new in the ultrastructure of tumor invasion *in vivo*? *Pathol. Res. Pract.*, **183**, 792–808 (1988).
33) Gusterson, B. A., Warburton, M. J., Mitchell, D., Kraft, N. and Hancock, W. W. Invading squamous cell carcinoma can retain a basal lamina. *Lab. Invest.*, **51**, 82–87 (1984).
34) Nöel, A. C., Callé, A., Emonard, H. P., Nusgens, B. V., Simar, N. L., Foidart, J., Lapiere, C. M. and Foidart, J. M. Invasion of reconstituted basement membrane matrix is not correlated to the malignant metastatic cell phenotype. *Cancer Res.*, **51**, 405–414 (1991).
35) Hynes, R. O. Integrins: a family of cell surface receptors. *Cell*, **48**, 549–554 (1987).
36) Clark, R. A. F., Nielsen, L. D., Howell, S. E. and Folkvord, J. M. Human keratinocytes that have not terminally differentiated synthesize laminin and fibronectin but deposit only fibronectin in the pericellular matrix. *In* "Extracellular Matrix: Structure and Function," ed. A. H. Reddi, pp. 247–261 (1985). Alan R. Liss Inc., New York.
37) D'Ardenne, A. J. and Barnard, N. J. Paucity of fibronectin in invasive lobular carcinoma of breast. *J. Pathol.*, **157**, 219–224 (1989).
38) Peltonen, J., Larjava, H., Jaakkola, S., Gralnick, H., Akiyama, S. K., Yamada, S. S., Yamada, K. M. and Uitto, J. Localization of integrin receptors for fibronectin, collagen, and laminin in human skin. *J. Clin. Invest.*, **84**, 1916–1923 (1989).
39) Ruoslahti, E. Integrins. *J. Clin. Invest.*, **87**, 1–5 (1991).
40) Murphy, G., Hembey, R. M., McGarrity, A. M. and Reynolds, J. J. Gelatinase (type IV collagenase) immunolocalization in cells and tissues: use of an antiserum to rabbit bone gelatinase that identifies high and low Mr forms. *J. Cell Sci.*, **92**, 487–495 (1989).
41) Liotta, L. A., Rao, C. N. and Barsky, S. H. Tumor invasion and the extracellular matrix. *Lab. Invest.*, **49**,

636–649 (1983).
42) Liotta, L. A. Tumor invasion and metastases — role of extracellular matrix: Rhoads memorial award lecture. *Cancer Res.,* **46,** 1–7 (1986).
43) Yamagata, S., Ito, Y., Tanaka, R. and Shimizu, S. Gelatinases of metastatic cell lines of murine colonic carcinoma as detected by substrate-gel electrophoresis. *Biochem. Biophys. Res. Commun.,* **151,** 158–162 (1988).
44) Ballin, M., Gromez, D. E., Shiha, C. C. and Thorgeirsson, V. P. Ras oncogene mediated induction of a 92kDa metalloproteinase; strong correlation with the malignant phenotype. *Biochem. Biophys. Res. Commun.,* **154,** 832–838 (1988).
45) Pyke, C., Ralfkiae, E., Huhtala, P., Hurskainen, T., Dano, K. and Trygvason, K. Localization of messenger RNA for Mr 72,000 and 92,000 type IV collagenases in human skin cancers by *in situ* hybridization. *Cancer Res.,* **52,** 1336–1341 (1992).
46) Okada, Y., Gonoji, Y., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K. and Hayakawa, T. Matrix metalloproteinase 9 (92 kDa gelatinase/type IV collagenase) from HT 1080 human fibrosarcoma cells. Purification and activation of the precursor, and enzymic properties. *J. Biol. Chem.,* **267,** 21712–21719 (1992).