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Potentiation of Metastatic Capacity by Transforming Growth Factor-β1 Gene Transfection

Noboru Ueki,[1,3] Toshihisa Ohkawa,[1] Yuji Yokoyama,[1] Juichiro Maeda,[1] Yutaka Kawai,[1] Tatsuhiko Ikeda,[2] Yoshiki Amuro,[1] Toshikazu Hada[1] and Kazuya Higashino[1]

[1]*The Third Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663 and* [2]*Morinaga Institute of Biological Science, 2-1-1 Shimosueyoshi, Tsurumi-ku, Yokohama, Kanagawa 230*

This study was designed to assess whether the excessive secretion of transforming growth factor-β1 (TGF-β1) by Chinese hamster ovary (CHO) cells transfected with TGF-β1 gene may be linked to the development of a metastatic phenotype. We observed large numbers of metastatic colonies in the lungs of nude mice inoculated with the transfected CHO cells. The tumors derived from these transfected cells demonstrated marked angiogenesis. We postulate that the overproduction of TGF-β1 by these tumors may participate in the metastatic progression following establishment of angiogenesis at the primary tumor site.

Key words: Transforming growth factor-β1 — Gene transfer — Angiogenesis — Lung metastasis

Transforming growth factor-β (TGF-β), a peptide first identified by its ability to cause phenotypic transformation of rat fibroblasts, exerts numerous regulatory actions in a variety of cells.[1)] Reported effects of TGF-β include the induction or inhibition of differentiation, the stimulation or inhibition of proliferation, and immunosuppression. Because most cells can produce as well as respond to TGF-β, this factor has particular significance in controlling complex physiological processes that involve interactions between different types of cells. The growth of most normal cells is inhibited by TGF-β, whereas their neoplastic counterparts are frequently, but not always, refractory to such inhibition.[1)] Many tumor cells also produce increased quantities of TGF-β that may promote tumor growth via the stimulation of tumor stroma formation and angiogenesis.[2, 3)]

There are currently three known mammalian TGF-β (β1, β2, and β3) genes.[1)] Although the potency of these three isoforms differs and they are regulated differently, they exhibit similar biological effects.[1)] TGF-β1, the first to be isolated, is also the best characterized. We previously demonstrated that TGF-β1 secreted by Chinese hamster ovary cells transfected with TGF-β1 cDNA could promote tumor cell proliferation *in vivo* by increasing the growth of stromal elements, including angiogenesis, through paracrine mechanisms.[3)] Although the aberrant production of TGF-β has been linked to the tumorigenicity of some transformed cells, its influence on metastatic progression is not fully understood. We designed the present study to determine whether the excessive secretion of TGF-β1 by transfectants may be linked to the development of a metastatic phenotype following the establishment of angiogenesis at the site of the primary tumor.

Chinese hamster ovary (CHO) cells deficient in dihydrofolate reductase (dhfr) activity were transfected with the amplifiable plasmid pSV2-(TGF-β-dhfr), which contains both the human TGF-β1 cDNA and mouse dhfr gene as well as intervening simian virus 40.[3)] The dhfr-amplified CHO cells were derived and adapted to increasing concentrations of methotrexate (MTX; Sigma Chemical Co., St. Louis, Mo.). Individual transfectant clones were selected, expanded, and screened for TGF-β1 production by the growth-inhibition assay of CCL-64 cells as described previously.[3)] The two dhfr-amplified transfectants (TIA and TIB) secreted very large amounts of TGF-β1 as a result of exposure to increasing concentrations of MTX (Table I). Since the proliferation of both the parental and transfected CHO clones was not stimulated or inhibited by TGF-β1 (0.1–50 ng/ml) or by the anti-TGF-β1 antibody (10–500 μg/ml), the recombinant TGF-β1 secreted by these transfected clones did not function as a negative autocrine growth factor (data not shown).

Balb/c nude mice (6-week-old female, CLEA Japan) were administered a subcutaneous injection of 2×10^6 cells of each cell line. Tumor volume was estimated as the product of three-dimensional measurements (length and width of longest surface and thickness of tumor determined with calipers) over a 5-week period. At different times after inoculation of each cell line, tumor tissues and organs were collected, fixed in 10% formalin, embedded

[3] To whom correspondence should be addressed.

Table I. TGF-β1 Secretion and Tumorigenicity of Transfected CHO Cells in Nude Mice

Clone	TGF-β1 secretion (ng/ml/24 h)	Tumor incidence	Tumor volume (mm^3)
Parental CHO	3.8[a)]	3/4	1109
Clone TIA	1750.0	4/4	12593
Clone TIB	1060.0	4/4	8933

The amounts of TGF-β1 produced was determined with a growth inhibition assay using CCL-64 cells and highly purified human platelet TGF-β1 as the standard. Both the tumor incidence and volume were determined at 4 weeks after inoculation with each cell line.
a) Data represent the average of triplicate experiments.

Table II. Angiogenic and Lung-colonizing Activity of Transfected CHO Cells in Nude Mice

Clone	No. of mice examined	Incidence of angiogenesis in primary tumor[a)]	Lung weight (g)	No. of lung metastases
Parental CHO	3	21±8[b)]	0.087±0.003	ND
Clone TIA	4	115±19	0.123±0.008	105±16
Clone TIB	4	96±15	0.138±0.012	117±20

Cells from each clone were subcutaneously injected into nude mice. All the mice were killed and examined at 4 weeks after injection.
a) The incidence of angiogenesis in the primary tumor per unit area is scored morphometrically using the hit-point method.[3] Eight areas of the peripheral region were observed for each tumor.
b) Data represent the mean±SD.
ND: not detected.

in paraffin, and stained with hematoxylin-eosin. The lungs were fixed and the metastatic colonies were counted under a dissecting microscope. Macroscopic observations of metastases were confirmed by histological methods. Both the TIA and TIB clones grew more rapidly than control cells when injected subcutaneously into nude mice (Table I). In addition, elevated levels of plasma TGF-β1 were observed in nude mice with both TIA and TIB tumors, but not in the plasma of animals with parental CHO tumors (data not shown). Histologically, marked angiogenesis was demonstrated in the tumors that were derived from two transfected clones, and there was no host cellular infiltration except for occasional lymphocytes or neutrophils (data not shown).

We next examined the relationship between the incidence of angiogenesis in the primary tumor and the development of lung metastases in the nude mouse model (Table II). We evaluated the incidence of angiogenesis in the primary tumor using morphometric techniques as previously described.[3] Eight different pictures of the histological section were taken at random for each tumor (magnification, ×240, one picture covered 0.285 mm^2). To count blood vessels, the picture was overlaid with a colorless sheet containing a dot matrix (28×17; 408 total). Dots which overlapped with capillaries were scored. The number of tumor colonies in lung was also counted under a dissecting microscope. The incidence of angiogenesis in both TIA and TIB primary tumors was significantly elevated as compared with the control tumors (Table II). Nude mice inoculated with the two transfectants had large numbers of metastatic colonies in the lungs which demonstrated angiogenesis, as did the tumors at the primary sites (Fig. 1). Most of the small metastatic colonies were observed in the pulmonary vasculatures, and formed large tumors characterized by the presence of capillaries in the lungs (Fig. 1D). In contrast, no pulmonary metastasis was found in the animals inoculated with parental CHO cells. These results suggested that pulmonary metastasis was correlated with angiogenesis in the primary tumor.

However, since tumor growth rates of each transfected clone were much higher than that of the parental CHO clone, it is unknown whether or not the metastatic potentials of these clones are independent of increased growth rate. To answer this question, we examined the number of pulmonary metastases and the incidence of angiogenesis at the same size of primary tumor. At 2 weeks after subcutaneous injection with TIA cells, metastases were found in the lungs of all mice, whereas no metastases of the lungs were observed, even at 5 weeks after injection with CHO cells (Table III). Histologically, sections of the primary tumors showed the formation of sinusoid-like capillaries in the presence of pericytes, mainly at the periphery of the tumor at 2 weeks after inoculation with TIA cells, compared with the tumor derived from CHO cells at 5 weeks after injection (Fig. 2). In the primary tumor derived from TIA cells, congestion and stasis of red blood cells, characteristic of new blood vessels,[4] were observed. In addition, most of the metastatic colonies were microscopically identified in pulmonary vessels in animals injected with TIA cells (Fig. 2). TIA cells or clumps are consequently considered to have a greater tendency to arrest in the pulmonary vasculatures and to have increased embolization. The ability of tumor cells to induce angiogenesis at the primary tumor site is considered to be one of the several steps required to establish the metastatic process.[5] Since the sections of tumors at the primary site demonstrated the formation of capillaries in our experiment, these tumor cells or clumps were considered to have spread via the bloodstream, i.e., exhibited hematogenous dissemination, and to have increased embolization, survival, and

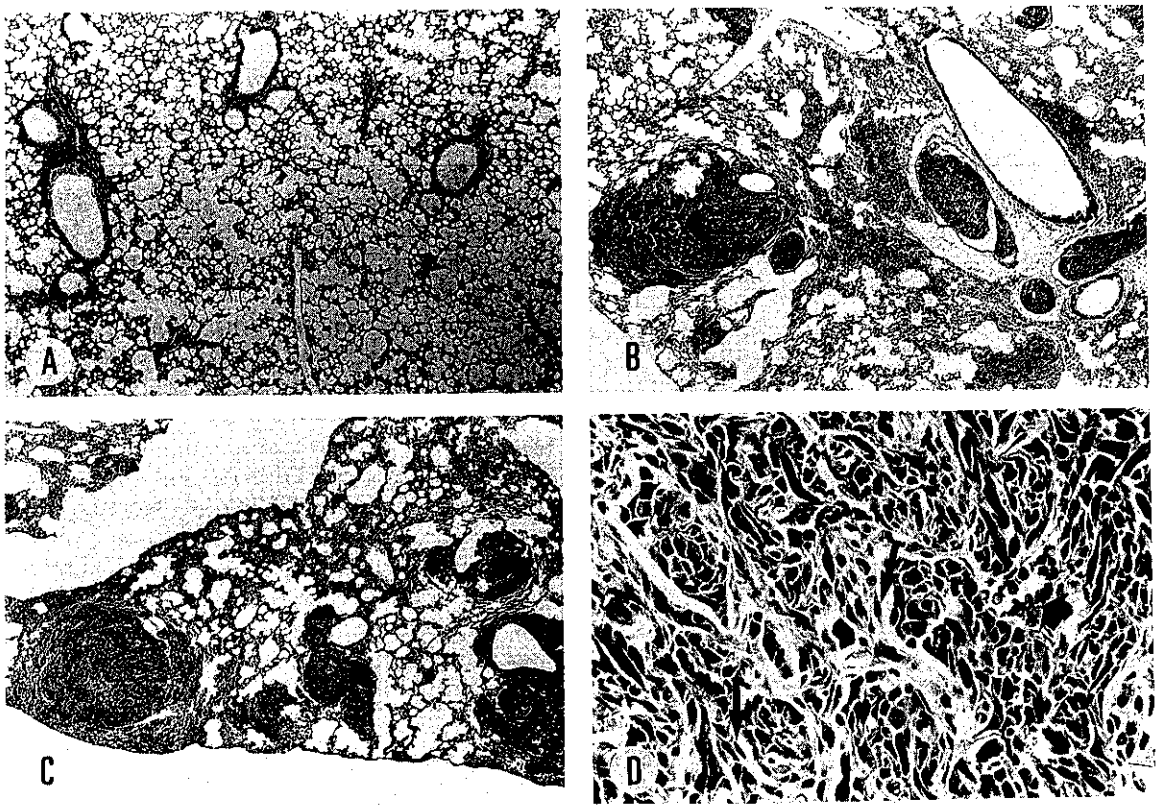

Fig. 1. Representative histology of pulmonary metastasis in nude mice that were subcutaneously inoculated with parental and two transfected CHO clones. (A) No pulmonary metastasis produced by parental CHO cells. (B, D) Pulmonary metastasis produced by TIA cells. (C) Pulmonary metastasis produced by TIB cells. A, B, C: ×50, D: ×480. Hematoxylin-eosin stain. Bold arrow indicates vessels in which red blood cells can be seen. Light arrows indicate the pericyte-like cells present in tumor metastasis in lungs.

Table III. Angiogenic and Lung-colonizing Activity of Transfected CHO Cells at the Same Size of Primary Tumors in Nude Mice

Clone	Time of excision (wk)	Tumor volume (mm^3)	Incidence of angiogenesis in primary tumor	No. of lung metastases
Parental CHO	5	1828±820[a)]	26±10	ND
Clone TIA	2	2079±956	75±16	17±5

Nude mice were given injections of cells from each clone. The tumors and lungs were excised from groups of 3 mice.
a) Data represent the mean±SD.
ND: not detected.

growth characteristics. Several lines of evidence support the concept that there may be substantial differences in the architecture of tumor vessels as compared with that of normal tissues.[4] It is conceivable that a variation in the character of the tumor vessels, which may present a poor barrier to tumor cell invasion, may facilitate the dissemination of tumors. TGF-β1 is stored in the greatest amount in platelets and is released from them during degranulation, and it has been suggested to play a major role in wound healing.[6,7] The platelet-aggregating ability of certain tumor cells has also been reported to have a crucial role in the formation of metastasis.[8,9]

Although it is important to study the activation mechanism of latent TGF-β1 to clarify the biological effects of this factor *in vivo*, the mechanism has not been fully elucidated. It is not easy to determine how much of the activated form of the total plasma TGF-β1 in nude mice bearing tumors is derived from transfected clones. A recent study suggests that the vascular pericytes are involved in the elaboration of activated TGF-β1 *in vivo*.[10] Since many pericytes were found in the sections of both the primary tumors and metastatic colonies in the lungs, these cells might be considered to act locally as an activator for the latent complex of TGF-β1. We therefore postulate that the enhanced production of TGF-β1

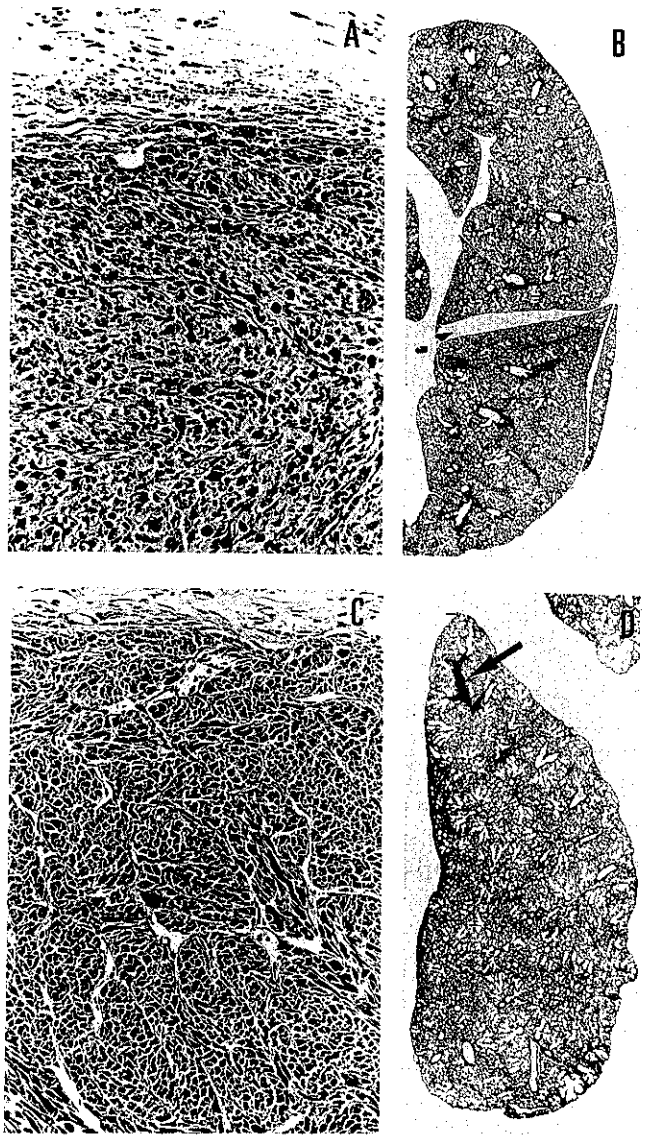

Fig. 2. Representative histology of sections of the primary tumors and lungs of nude mice that were subcutaneously inoculated with CHO and TIA cells. (A, B) At 5 weeks after injection of parental CHO cells (A, primary tumor, ×240; B, lung, ×10). (C, D) At 2 weeks after injection of TIA cells (C, primary tumor, ×240; D, lung, ×10). Hematoxylin-eosin stain. Arrow indicates metastatic colonies in the pulmonary vasculature.

by the tumors may be associated with the development of metastatic phenotype following the establishment of angiogenesis at the primary site. Liotta *et al.* showed that the number of pulmonary metastases was correlated with the number of blood vessels in the tumor.[11] Our findings also suggest that there is a correlation between the occurrence of angiogenesis and the incidence of metastasis.

TGF-β1 may influence metastatic cell growth via complex pathways. TGF-β1 modulates the synthesis of several proteases and protease inhibitors in a variety of cell types.[12, 13] Following transformation, proteases are often constitutively expressed at high levels. The observation that malignant fibrosarcomas secrete high levels of TGF-β1 raises the possibility that these tumors may maintain an increased production of protease via the autocrine effects of TGF-β1.[14] *In vitro* studies recently showed that induction of TGF-β1 secretion can enhance the cellular motility and the production of protease, thus increasing the invasive potential of fibrosarcomas.[15] TGF-β1 also influences the synthesis of matrix proteins and their cellular receptors,[16, 17] events that may also be important in tumor cell invasion and metastasis *in vivo*.[18] However, the information gleaned from analysis of cell lines might not properly reflect the *in vivo* actions of TGF-β in the development of a metastatic phenotype. Recently other investigators have reported on the role of TGF-β1 in modifying the invasive and metastatic potential of tumor cells *in vivo*.[19, 20] Since TGF-β1 is an important modulator of angiogenesis, and increases production of basement membrane-degrading enzymes, this factor may alter the cellular milieu in such a way as to favor metastatic spread.

In this study, we found that the progression of metastasis was associated with an increase in TGF-β1 secretion, which in turn was intimately linked to angiogenesis at the primary site. The transfected clones and/or tumors used in this study may provide suitable models for further studies on the development of the metastatic phenotype by enhanced secretion of TGF-β1.

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