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Stimulation of the Growth of Metastatic Clones of Mouse Colon Adenocarcinoma 26 *in vitro* by Platelet-derived Growth Factor

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The induction of platelet aggregation by tumor cells was found to be an important determinant for the formation of metastasis of a highly metastatic variant of mouse colon adenocarcinoma 26. We found that the growth of highly metastatic clones, NL-17 and NL-33, of mouse colon 26 was well stimulated by platelet-derived growth factor (PDGF) and the stimulation was dependent on the concentration of the growth factor. The growth of weakly metastatic clones, NL-4 and NL-44, was stimulated marginally by PDGF. Other factors such as transforming growth factor β and epidermal growth factor did not stimulate the growth of metastatic clones. As the amounts of the receptor of PDGF, as determined by [^{125}I]PDGF binding and mRNA expression, were almost equal in NL-17 and NL-44 clones, the difference in growth potential of these clones after the treatment with PDGF could be explained by post-receptor mechanism(s). The present findings indicate that when tumor cells are arrested in a capillary through the formation of aggregates with platelets, PDGF might play an important role in the establishment of metastasis of mouse colon 26.

Key words: Growth factor — Platelet-derived growth factor — PDGF receptor — Metastasis

Formation of metastasis is influenced by various factors from host cells and tumor cells.[1-5] We have established various high- and low-metastatic clones from a metastatic vatiant of mouse colon adenocarcinoma 26.[6] We found that the induction of platelet aggregation *in vitro* significantly increased the formation of pulmonary metastasis of a highly metastatic clone, NL-17, of colon 26.[7] The growth of the clone NL-17 is stimulated well by the extract from the lung.[8] Obviously the growth potential of the cells is an important determinant for the formation of metastasis.[8, 9] As the NL-17 clone induces platelet aggregation and forms a massive metastasis *in vivo*, we examined the effect of platelet-derived growth factor (PDGF) on the proliferation of NL-17 as well as other metastatic clones derived from mouse colon adenocarcinoma 26. We found that highly metastatic tumor cells respond well to PDGF. These results indicate that platelet aggregation induced by metastatic tumor cells followed by the response of tumor cells to PDGF might be an important process in the formation of experimental metastasis of colon adenocarcinoma 26.

MATERIALS AND METHODS

Metastatic tumor cells Metastatic mouse colon adenocarcinoma 26 clones, the low-metastatic NL-4, NL-14 and NL-44, and the high-metastatic NL-17 and NL-33 in the case of iv inoculation, and the high-metastatic NL-22 in the case of sc inoculation, were used in this study. These clones were established in our laboratory from a metastatic variant of the colon adenocarcinoma 26 as described previously.[6]

Metastatic mouse B16 melanoma and K1735 melanoma clones, the low-metastatic B16 F1, and K1735 C110 and C123, and the high-metastatic B16 F10 and K1735 M2 in the case of iv inoculation, and the high-metastatic clone B16 BL-6 in the case of sc inoculation, were kindly provided by Dr. I. J. Fidler. Tumor clones were maintained in α-MEM supplemented with 5% calf serum and 3% fetal bovine serum (growth medium) at 37°C in a humidified atmosphere of 5% CO_2.

Growth factors PDGF from human platelets and epidermal growth factor (EGF) from mouse submaxillary glands were the products of Collaborative Research Inc. (Lexington, MA). Highly purified PDGF was kindly provided Dr. Kaji, Tokyo Metropolitan Institute of Gerontology. Transforming growth factor β (TGF-β) was kindly provided by Dr. Hirai, Tokyo Institute of Medical Sciences. [^{125}I]PDGF (1,024 Ci/mmol) was obtained from Amersham Japan.

Assay of growth-stimulating activity Growth-stimulating activity was measured according to the method previously reported.[7] Briefly, tumor cells were suspended in RPMI 1640 medium containing 1% fetal bovine serum and plated at concentration of 5×10^4 cells/well in 12-well plates (Costar, Cambridge, MA). After incubation for 24 h at 37°C, the attached cells were washed twice with RPMI 1640 medium containing 10 m*M* HEPES buffer (pH 7.4) without fetal bovine serum (serum-free RPMI), and fed with 2 ml of serum-free RPMI. Growth factor (final concentration 0.25–25 ng/ml) was dissolved and diluted in 0.1% (w/v) bovine serum albumin (fatty acid-free, Sigma, St. Louis, MO) solution in phosphate-buffered saline (pH 7.4) and was added to each culture to

give the final concentrations as indicated in the figure. The final concentration of bovine serum albumin (BSA) in each assay was 5 μg/ml. Tumor cells were counted after 4 days with a Model ZBI Coulter counter. Growth index was determined by dividing the cell counts of experimental cultures by the cell count of the control culture to which no growth factor had been added.

Binding assay Binding assays were performed as described previously.[10, 11] Briefly, NL-17 and NL-44 cells were plated at a density of 1×10^5 cells/well in 24-well plates (Corning, New York) and incubated for two days. The culture media were changed to fresh RPMI 1640 medium containing 0.04% BSA and 10 m*M* HEPES (pH 7.4) and the cells were incubated for 8 h. Following two washes with phosphate-buffered saline containing 0.9 m*M* $CaCl_2$ and 0.8 m*M* $MgSO_4$, the cells were incubated with various concentrations of [^{125}I]PDGF (0.01–0.16 pmol/ml) in 0.2 ml of the above buffer containing 0.25% BSA at 4°C for 2 h. For the correction of nonspecific binding, the cells were preincubated with a 100-fold concentration of unlabeled PDGF at 4°C for 1 h. After 3 washes with the buffer containing 1% BSA, the cells were lysed with 1% Triton X-100, 0.01% BSA, 10% glycerol and 2 m*M* HEPES (pH 7.4),[11] and the amount of bound [^{125}I]PDGF was determined in an Beckman autogamma counter.

RNA blot analysis Total cellular RNA was extracted from NL-17 and NL-44 cells according to the method of Maniatis *et al.*[12] employing guanidine thiocyanate extraction and cesium chloride centrifugation. Polyadenylated mRNA was isolated through an oligo(dT) column, and the mRNA (10 μg) was denatured, run on a 1% agarose gel in 40 m*M* 3-(N-morpholino)propanesulfonic acid-10 m*M* sodium acetate-1 m*M* EDTA, pH 7.0, containing 2.2 *M* formaldehyde, and transferred to a nitrocellulose filter.[13] The filter was dried, baked, and hybridized with ^{32}P-labeled mouse cDNA probe for the receptor of platelet-derived growth factor as described previously.[13] cDNA probe for the receptor of platelet-derived growth factor was kindly provided by Dr. Y. Yarden, Genentech Inc., South San Francisco, CA.[14]

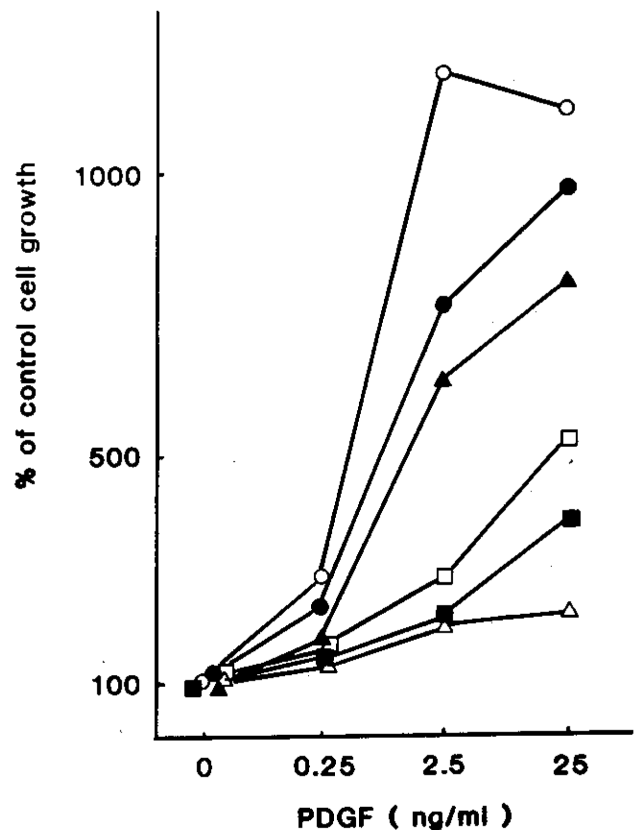

Fig. 1. Growth stimulation of metastatic clones of mouse colon adenocarcinoma 26 by PDGF. Metastatic tumor cells were seeded at 5×10^4 cells/well (Costar 12-well plate) for 24 h at 37°C, washed twice with RPMI 1640 medium without serum and fed with 2 ml of serum-free RPMI 1640 containing PDGF as indicated. The cell number was counted 4 days later in triplicate and the relative cell number as compared to the control (without PDGF) culture was plotted in the figure. ■, NL-4; ▲, NL-14; ○, NL-17; □, NL-22; ●, NL-33; △, NL-44.

RESULTS

Stimulation of the growth *in vitro* by PDGF The effect of PDGF on the proliferation of metastatic mouse colon 26 clones was examined (Fig. 1). The growth was generally and reproducibly stimulated depending on the concentration of PDGF. Obviously the highly metastatic NL-17 and NL-33 were stimulated very well by PDGF and a 7.5- to 12-fold increase in cell number was observed with 2.5–25 ng/ml of PDGF. On the contrary, the growth of weakly metastatic NL-4 and NL-44 was stimulated marginally by PDGF; 2- to 3.5-fold growth stimulation occurred with PDGF at 2.5–25 ng/ml. The growth of NL-14 was stimulated 6- to 8-fold. NL-14 is weakly metastatic because of its inability to induce platelet aggregation after iv inoculation.[9] NL-22 is a highly metastatic variant when inoculated sc.[6] The growth of NL-22 was moderately stimulated, and a 3- to 5-fold increase in cell number was observed with 2.5–25 ng/ml of PDGF.

PDGF, however, did not significantly stimulate the growth of metastatic clones derived from K1735 melanoma and B16 melanoma. Among the clones, the growth of highly metastatic clones K1735 M2 and weakly metastatic B16 F1 was only marginally stimulated by PDGF (Fig. 2A and 2B).

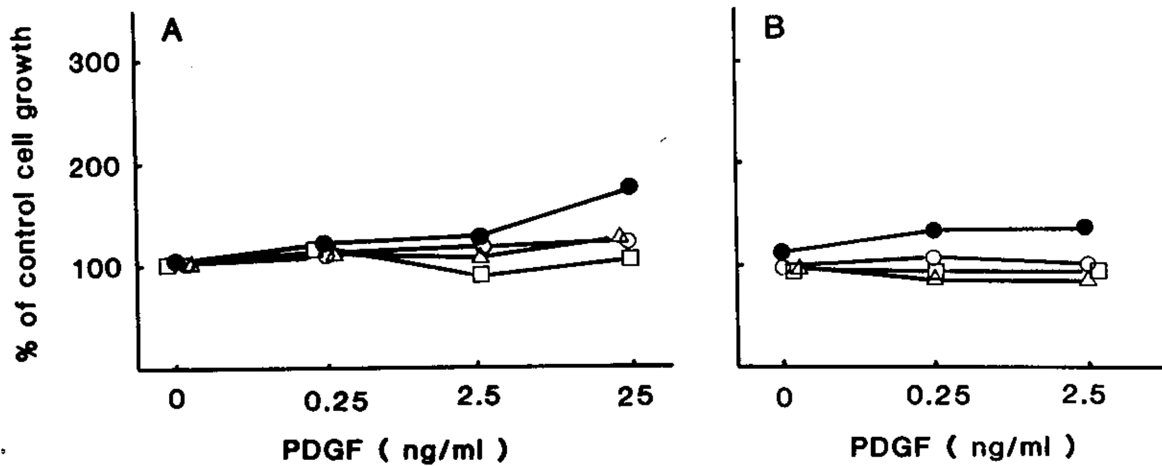

Fig. 2. Effect of PDGF on the growth of metastatic clones of K1735 and B16 mouse melanomas. The same experiment as described in Fig. 1 was carried out with K1735 melanoma parent (○), its highly metastatic clone M2 (●) and weakly metastatic clones C110 (△) and C123 (□) in A, and with B16 melanoma parent (○), its weakly metastatic clone F1 (●) and highly metastatic clones F10 (△) and BL-6 (□) in B.

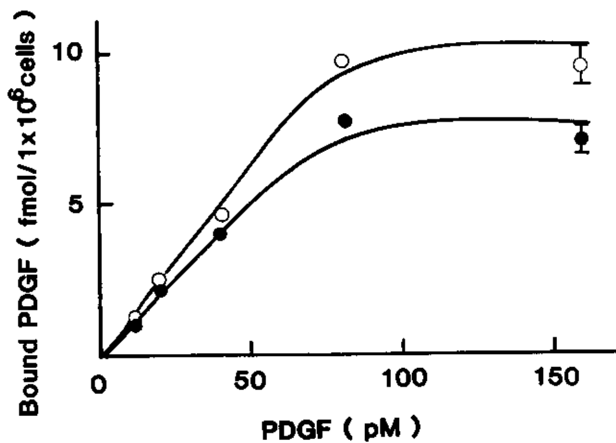

Fig. 3. Binding of PDGF to NL-17 and NL-44 cells. The binding of [^{125}I]PDGF was examined with NL-17 (○) and NL-44 (●) cells under the conditions described in "Materials and Methods." The standard deviation of three determinations of each point was within 4% except for those shown by a bar.

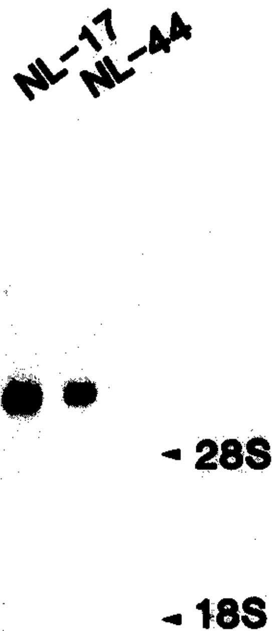

Fig. 4. Northern blot analysis of PDGF receptor mRNA. mRNA was run on a 1% agarose gel and transferred to a nitrocellulose filter as described in "Materials and Methods." The RNA was hybridized with ^{32}P-labeled mouse cDNA probe for receptor of platelet-derived growth factor.

Effect of TGF-β and EGF on the growth of metastatic clones TGF-β is known to be released from platelets after stimulation. TGF-β, however, did not stimulate the growth of NL-17 or NL-44, EGF also had no effect on the growth of other NL-clones. The combination of PDGF at a marginally effective concentration (0.25 ng/ml) with EGF did not induce stimulation of growth. Rather, a slight inhibitory effect occurred with NL-17 when the concentration of EGF was increased.

PDGF receptor of NL-17 and NL-44 To analyze the mechanisms of the different responses of NL-17 and NL-44 clones to PDGF, we measured the number of PDGF receptors of the cells. As no monoclonal antibody for PDGF receptor was available, we measured the level of the binding of PDGF and the mRNA expression of the receptors by using NL-17 and NL-44 clones.

The binding of [^{125}I]PDGF to the cells increased depending on the concentration of PDGF (Fig. 3). Maximal binding occurred above 80 p*M* PDGF under the experimental conditions used. Scatchard analysis revealed that NL-17 and NL-44 has similar numbers of PDGF binding sites (receptors) which had similar dissociation constant values: NL-17 16 fmol/10^6 cell, *Kd* 81 p*M*; NL-44 11 fmol/10^6 cell, *Kd* 65 p*M*. Furthermore, as shown in Fig. 4, no quantitative difference was found in the amount of mRNA of PDGF receptors between NL-17 and NL-44. These results indicate that the difference in growth promotion by PDGF between NL-17 and NL-44 cells could not be explained by the number of PDGF receptors. The different responses of NL-17 and NL-44 cells to PDGF might be explained by postreceptor mechanisms.

DISCUSSION

There are many possible approaches to the study of tumor metastasis. We focused our interest on the arrest of circulating tumor cells, and especially on the subsequent growth of tumor cells. The formation of metastatic nodules is often influenced by the observation period.[9] The proliferation rate of tumor cells *in vivo* is obviously an important factor affecting formation of tumor metastasis. Several factors could be involved in the *in vivo* growth of metastatic cells; for example, growth-promoting activities from homing cells, certain growth factors in the bloodstream, and some growth factors derived from host cells could play important roles (see refs. 4 and 5 for reviews, and ref. 15).

The highly metastatic variants of mouse colon 26 used in this experiment possess rather strong platelet-aggregating activity, which is an important factor affecting the formation of tumor metastasis as reported previously.[7, 9] These observations prompted us to examine the response of the metastatic variants to the platelet-derived growth factor. Among the clones we examined, two highly metastatic variants NL-17 and NL-33 with platelet-aggregating activity responded well to PDGF, and two weakly metastatic variants NL-4 and NL-44 with platelet-aggregating activity responded rather poorly to PDGF (Fig. 1). These findings might indicate that growth stimulation of tumor cells by PDGF is also an important factor influencing the formation of metastasis of mouse colon adenocarcinoma 26.

NL-14 also responded well to PDGF, but the metastatic ability of this clone is rather low. NL-14 lacks the platelet-aggregating activity. When the clone NL-14 was fused with a clone having platelet-aggregating activity, the hybrid cells became metastatic.[9] Thus, NL-14, in spite of its good response to PDGF, is weakly metastatic because of its inability to induce platelet aggregation. NL-22 is metastatic to the lung when the clone was inoculated sc. The metastatic ability of NL-22 after iv inoculation was lower than that of NL-17 and NL-33.[6] NL-22 responded moderately to PDGF. The good response of tumor cells to PDGF seems not to be necessarily important for the formation of spontaneous (from sc route to the lung) metastasis. The present findings indicate, however, that the response of the tumor cells to PDGF is an important determinant for the successful formation of experimental (from iv route to the lung) metastasis in the case of mouse colon adenocarcinoma 26. Other growth factors such as TGF-β and EGF were not effective in this system, indicating the specificity of the stimulation by PDGF in the present system.

The present phenomenon, however, was not observed in metastatic variants from B16 and K1735 melanomas. In these tumor variants, other factors might influence the formation of metastasis even after iv inoculation. These observations indicate the diversity of the process of tumor metastasis, and also indicate the heterogeneity of metastatic abilities of tumor cells within a single tumor and among different tumors.

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REFERENCES

1) Fidler, I. J., Gersten, D. M. and Hart, I. R. The biology of cancer invasion and metastasis. *Adv. Cancer Res.*, **28**, 149–250 (1978).
2) Roos, E. and Dingemans, K. P. Mechanism of metastasis. *Biochim. Biophys. Acta*, **560**, 135–166 (1979).
3) Paste, G. and Fidler, I. J. The pathogenesis of cancer metastasis. *Nature*, **283**, 139–146 (1980).
4) Nicolson, G. L. Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. *Cancer Metastasis Rev.*, **7**, 143–188 (1988).
5) Nicolson, G. L. Cancer metastasis: tumor cell and host organ properties important in metastasis to specific secondary sites. *Biochim. Biophys. Acta* (1988), in press.
6) Tsuruo, T., Yamori, T., Naganuma, K., Tsukagoshi, S. and Sakurai, Y. Characterization of metastatic clones derived from a metastatic variant of mouse colon adenocarcinoma 26. *Cancer Res.*, **43**, 5437–5442 (1983).
7) Tsuruo, T., Kawabata, H., Iida, H. and Yamori, T. Tumor-induced platelet aggregation and growth promoting factors as determinants for successful tumor metastasis. *Clin. Exp. Metastasis*, **4**, 25–33 (1986).
8) Yamori, T., Iida, H., Tsukagoshi, S. and Tsuruo, T. Growth stimulating activity of lung extract on lung-colonizing colon 26 clones and its partial characterization. *Clin. Exp. Metastasis*, **6**, 131–139 (1988).
9) Sugimoto, Y., Oh-hara, T., Watanabe, M., Saito, H., Yamori, T. and Tsuruo, T. Acquisition of metastatic ability in hybridomas between two low metastatic clones of murine colon adenocarcinoma 26 defective in either platelet-aggregating activity or *in vivo* growth potential. *Cancer Res.*, **47**, 4396–4401 (1987).
10) Koenuma, M., Yamori, T. and Tsuruo, T. Insulin and insulin-like growth factor 1 stimulate proliferation of metastatic variants of colon carcinoma 26. *Jpn. J. Cancer Res.*, **80**, 51–58 (1989).
11) Heldin, C-H., Wasteson, A. and Westermark, B. Interaction of platelet-derived growth factor with its fibroblast receptor. *J. Biol. Chem.*, **257**, 4216–4221 (1982).
12) Maniatis, T., Frisch, E. F. and Sambrook, J. "Molecular Cloning. A Laboratory Manual," pp. 196 (1982). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
13) Sugimoto, Y. and Tsuruo, T. DNA-mediated transfer and cloning of a human multidrug-resistant gene of adriamycin-resistant myelogenous leukemia K562. *Cancer Res.*, **47**, 2620–2625 (1987).
14) Yarden, Y., Escobedo, J. A., Kuang, W-J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Francke, U., Friend, V. A., Ullrich, A. and Williams, L. T. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. *Nature*, **323**, 226–232 (1986).
15) Rodeck, U., Herlyn, M., Menssen, H. D., Furlanetto, R. W. and Koprowski, H. Metastatic but not primary melanoma cell lines grow *in vitro* independently of exogenous growth factors. *Int. J. Cancer*, **40**, 687–690 (1987).