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Insulin and Insulin-like Growth Factor 1 Stimulate Proliferation of Metastatic Variants of Colon Carcinoma 26

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The proliferation rate of malignant cells *in vivo* is one of the important factors which affect the formation of tumor metastasis. A highly metastatic variant of mouse colon adenocarcinoma 26 (NL-17) grew more rapidly than a low-metastatic variant (NL-44) both *in vitro* and *in vivo*. The effect of growth factors on the proliferation of NL-17 and NL-44 cells was examined in serum-free medium. Among growth factors examined, human insulin and insulin-like growth factor 1 (IGF-1), which were produced by gene engineering techniques, stimulated the growth of metastatic NL-17 and NL-44 cells as determined by thymidine incorporation and cell counts. DNA synthesis and cell proliferation of the high-metastatic NL-17 was stimulated to a greater extent by insulin and IGF-1 than those of the low-metastatic NL-44. These findings suggest that circulating growth factors could enhance the formation of tumor metastasis. Scatchard analysis of [^{125}I]IGF-1 binding to NL-17 and NL-44 showed that each cell line had an almost equal number of IGF-1 receptors (1.37×10^5/cell and 1.26×10^5/cell, respectively), which had similar dissociation constants (8.94×10^{-10} *M* and 9.54×10^{-10} *M*, respectively). Since the number and affinity of IGF-1 receptors are equivalent between low- and high-metastatic cells, the intracellular events which result in the cell growth after binding of IGF-1 may differ between NL-17 and NL-44 cells.

Key words: Metastasis — Growth factor — IGF-1 — Insulin — Colon carcinoma 26

Tumor metastasis is a complex, multiple-step process that includes: detachment of tumor cells from the primary tumor; invasion into the host's blood vessel; transportation in the blood stream; adhesion to a vessel wall of a distinct organ; and proliferation at the secondary site.[1–3] The importance of each step is well documented. We have been interested in the arrest of circulating tumor cells,[4] and their subsequent growth at distant organ sites.[5, 6] In this context, the rate of tumor cell proliferation *in vivo* is an important factor which affects the formation of tumor metastases.

We have established metastatic variants of mouse colon adenocarcinoma 26 after *in vivo* selection for lung-colonizing ability and subsequent *in vitro* cloning.[7] The high-metastatic variant, NL-17, develops many large nodules after iv injection while a low-metastatic variant, NL-44, forms a few small nodules in the lung. This system is good for analyzing the lung-colonizing process of tumor cells injected iv. No significant difference exists between NL-17 and NL-44 in their ability to induce platelet aggregation, which is a determinant of colon adenocarcinoma metastasis.[4, 5] Therefore the difference in metastatic potential between these two variants appears to depend on additional factors. In comparison to NL-44 cells, the NL-17 cells grow more rapidly when injected sc[6] and are stimulated to a greater extent for *in vitro* growth by lung-associated growth factors.[5] These observations indicated that tumor cell growth in the secondary organ may be an additional determinant of metastasis and, therefore, the growth factors provided in the environments of the secondary organ might affect the development of metastasis.

In this study, we examined the growth-stimulating effect of various growth factors which are known to exist in the circulation on the two different metastatic variants, NL-17 and NL-44. We found that the growth of NL-17 cells was better stimulated by human insulin and IGF-1[2] than that of NL-44 cells. These factors, especially IGF-1, might play an important role in the formation of metastases of NL-17 in the lung.

MATERIALS AND METHODS

Chemicals and reagents Recombinant human insulin (1,000 U = 3.7 mg) was kindly provided by Shionogi & Co., Ltd. Osaka. Recombinant human IGF-1 (somatomedin C), rat MSA, mouse EGF, bovine basic FGF and hydrocortisone were obtained from Collaborative Research, Inc. (Lexington, Mass.). Fatty acid-free

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[2] The abbreviations used are: IGF-1, insulin-like growth factor 1; MSA, multiplication stimulating activity; EGF, epidermal growth factor; FGF, fibroblast growth factor; BSA, bovine serum albumin; [^{125}I]insulin, [^{125}I]-(A14)-monoiodinated insulin; [^{125}I]IGF-1, (3-[^{125}I]iodotyrosyl)insulin-like growth factor-1; [^{3}H]thymidine, [methyl-^{3}H]thymidine; TGF$_\beta$, transforming growth factor β; KRP, Krebs-Ringer phosphate buffer; MEM, minimum essential medium; EC_{50}, effective concentration of non-labeled growth factor which inhibits the specific binding of labeled growth factor by 50% of the maximum.

BSA, gelatin and rat transferrin were obtained from Sigma (St. Louis, Mo.). [Methyl-^{3}H]thymidine (91 Ci/mmol), [^{125}I]insulin (2,000 Ci/mmol) and [^{125}I]IGF-1 (2,000 Ci/mmol) were obtained from Amersham Japan, Ltd. (Tokyo). Human TGF$_\beta$ was a gift from Dr. K. Kaji, Tokyo Metropolitan Institute of Gerontology (Tokyo).

Tumor cells Two tumor clones, the low-metastatic NL-44 and the high-metastatic NL-17, were established in our laboratory from a metastatic variant of the colon adenocarcinoma 26 as previously described.[7] Tumor clones were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. H35 cells were obtained from Professor M. Kasuga, Faculty of Medicine, University of Tokyo (Tokyo), and maintained in MEM containing 10% calf serum and 2% fetal bovine serum.

Assay of growth-promoting activity Subconfluent tumor cells were detached from a dish by a brief treatment with 0.01% trypsin containing 0.005% EDTA. The cells were suspended in RPMI 1640 medium containing 1% fetal bovine serum and plated at a concentration of 5×10^4 cells/ 2 ml/well in 12-well plates (Coster, Cambridge, Mass.).[5] These plates were precoated with 0.3% gelatin for 24 h and washed twice with Ca/Mg-free PBS. After incubation for 24 h at 37°C, the cell monolayers were washed twice with RPMI 1640 medium containing 10 m*M* Hepes buffer without fetal bovine serum and refed with RPMI 1640 medium containing 0.04% BSA and 10 m*M* Hepes buffer. At the time of medium change (day 1), cell numbers were counted in triplicate cultures. Growth factors were added to the remaining cultures and the number of tumor cells was counted after the incubation periods described in the legends. Growth-promoting activity was expressed by the ratio of the cell counts over day 1 cell counts. Cell counts were done by a model ZBI Coulter counter. All experiments were performed in triplicate.

Measurement of [^{3}H]thymidine incorporation Plates of NL-17 and NL-44 cells were prepared as above.[5] On day 1, the medium was changed to serum-free RPMI 1640 containing 0.04% BSA and 10m*M* Hepes buffer and the cells were incubated for an additional two days. On day 3, the medium was changed and the incubation was continued with or without growth factors in the medium. After 24 h, 1 μCi of [^{3}H]thymidine (91 Ci/mmol) per well was added and the plates were incubated for 2 h.[8] Wells were washed twice with Ca/Mg-free PBS and the cells were harvested with 0.05% trypsin containing 0.02% EDTA in Ca/Mg-free PBS. The cells were precipitated with 10% TCA and poured onto glass fiber filters. The filters were dried, and the radioactivity was counted in a liquid scintillation counter.

Binding assays NL-17 and NL-44 cells were suspended in RPMI 1640 mediun containing 10% fetal bovine serum. H35 cells were suspended in MEM containing 10% calf serum and 2% fetal bovine serum.[9] These cells were plated at a concentration of 1×10^5 cell/ml/well in 24-well plates (Corning, New York) and incubated for two days. The culture media were changed to fresh RPMI 1640 (for colon cells) or MEM medium (for H35 cells) containing 0.04% BSA and 10m*M* Hepes buffer and the cells were continuously incubated for 8 h. Semi-confluent cells were washed twice with ice-cold binding (KRP) buffer (125 m*M* NaCl, 5.2 m*M* KCl, 10 m*M* Na_2HPO_4, 1.4 m*M* $CaCl_2$ and 1.4 m*M* $MgSO_4$) containing 0.1% BSA. When the saturation of insulin receptor was examined, the cells were washed once with 0.2 *M* sodium acetate, pH 5.5 containing 0.4 *M* NaCl for 5 min,[10] and then washed twice with cold KRP buffer containing 0.1% BSA. The monolayer cells were incubated with 0.2 ml of binding buffer containing ^{125}I-labeled growth factors (0.1 ng of [^{125}I]insulin, 1.0×10^5 dpm/well; 0.1 ng of [^{125}I]IGF-1, 7.0×10^4 dpm/well) together with serial dilutions of unlabeled growth factor at 4°C for 15 h.[11] Nonspecific binding was determined by including 4 μg/well of cold insulin or 0.6 μg/well of cold IGF-1 in the binding mixture. The dishes were washed three times with ice-cold KRP buffer. Cell-associated radioactivity was determined after the lysis of the cells with Ca/Mg-free PBS containing 1% Triton X100, 0.01% BSA and 10% glycerol. The number of cells in duplicate wells was determined by a model ZBI Coulter counter.

Statistical analysis Student's *t* test was used for statistical analysis.

RESULTS

Effect of growth factors on the proliferation of NL-17 and NL-44 cells The effects of various growth factors at commonly used concentrations on the proliferation of NL-17 and NL-44 cells were examined under serum-free conditions (Table I). Human IGF-1 and human insulin stimulated the cell growth of NL-17 and NL-44, and the stimulative effects were significantly larger in NL-17 than in NL-44. At 1/3 and 1/9 of the concentrations of these growth factors used here, stimulative effects were also observed, but the extents were less than those described in the table. EGF, bovine basic FGF, rat MSA, rat transferrin or hydrocortisone did not stimulate the proliferation of NL-17 or NL-44. The effects of insulin and IGF-1 were further examined. The doubling times of NL-17 and NL-44 cells in the mudium supplemented with 10% calf serum were almost equal.[7]

Growth-stimulating effect of IGF-1 and insulin on NL-17 and NL-44 cells IGF-1 and insulin stimulated the proliferation of NL-17 cells in a dose-dependent manner (Fig. 1A and B). Maximum stimulation was achieved at either around 20 ng/ml of IGF-1 or 100 ng/ml of insulin

Table I. Response of NL-17 and NL-44 Cells to Growth Factors in Serum-free Medium

Addition	Concentration	Growth (% of control)[a]	
		NL-17	NL-44
None (control)	—	100±5.8	100±4.0
Insulin	100 ng/ml	510±9.3	282±8.9
IGF-1	10 ng/ml	490±2.8	279±3.4
EGF	10 ng/ml	102±3.0	101±4.2
TGF_β	5 ng/ml	100±6.3	95±5.0
MSA	40 ng/ml	117±6.8	102±4.0
Basic FGF	2 ng/ml	87±7.0	38±5.4
Rat transferrin	12 ng/ml	93±12.0	120±10
Hydrocortisone	500 n*M*	107±7.3	81±7.9

At day 1 growth factors were added to serum-free RPMI 1640 containing 0.04% BSA. The cells were counted on day 5 and compared to the control culture, to which no growth factors had been added. The numbers represent mean±SD of triplicate assays.
a) Values are mean±SD.

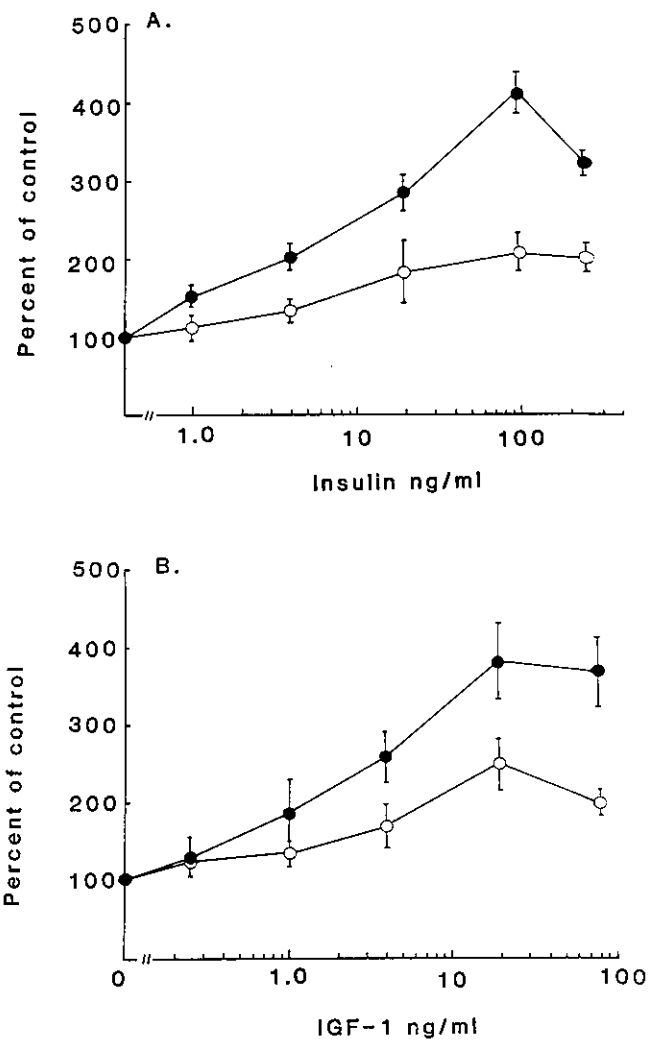

Fig. 1. The dose-dependent growth stimulation by insulin (A) and IGF-1 (B). The cells were plated on day 0, and serum-free culture supplemented with insulin or IGF-1 was started on day 1 as described in "Materials and Methods." The results are the averages of triplicate assays, and are expressed as a percent of cell number in the serum-free medium. NL-17, closed symbols (●); NL-44, open symbols (○).

to the level of more than 400% of the control growth. NL-44 cells were less sensitive to growth stimulation by IGF-1 and insulin than NL-17 cells. Maximum stimulation was observed at around 20 ng/ml IGF-1 or 100 ng/ml of insulin, but amounted to only about 250% and 200% of the control growth, respectively. At the highest concentrations of IGF-1, we observed slight decreases in growth stimulation of NL-17 and NL-44 cells, although the differences of the growth were not significant. These decreases might be caused by a down-regulation mechanism invoked by the addition of an excess amount of IGF-1, but more precise analysis is needed to reach a conclusion. As the stimulation of proliferation of NL-17 cells was nearly the same at 10 and 20 ng/ml of IGF-1 in repeated experiments (data not shown), an IGF-1 concentration of 10 ng/ml was used as the optimum for further experiments.

The stimulating effects of IGF-1 and insulin were confirmed by examining their DNA synthesis-stimulating activity (Table II). IGF-1 and insulin stimulated the DNA synthesis of NL-17 and NL-44 cells, and the stimulation was significantly greater in NL-17 cells than in NL-44 cells.

We examined the kinetics of growth stimulation of NL-17 and NL-44 cells by IGF-1 and insulin. In serum-free medium, NL-17 of NL-44 cells did not proliferate and no significant detachment during the five-day culture period was observed (Figs. 2 and 3). Both NL-17 and NL-44 initiated proliferation after the addition of 100 ng/ml of insulin or 10 ng/ml of IGF-1 on day 1. When 100 ng/ml of insulin was added, the doubling times of NL-17 and NL-44 were 40 h and 70 h, respectively. When 10 ng/ml of IGF-1 was added, the doubling times of NL-17 and NL-44 were 43 h and 102 h, respectively. The numbers of NL-17 and NL-44 cells (approximately 4.5×10^4 cells/well on day 1) increased to 2.2×10^5 cells/well and 1.3×10^5 cells/well, respectively, on day 5 after the addition of insulin. Similar increase in the cell numbers of NL-17 and NL-44 were observed after the addition of IGF-1. These data clearly indicate that insulin and IGF-1 stimulate the cell growth of high-metastatic NL-17 more than that of the low-metastatic NL-44.

Table II. Effect of Insulin and IGF-1 on [^{3}H]Thymidine Incorporation into NL-17 and NL-44

Growth factor concentraton (ng/ml)		Stimulated incorporation of [^{3}H]thymidine (pmol/5×10^4 cell)[a]		$P^{b)}$
		NL-17	NL-44	
Insulin	0	0	0	—
	1	0.6±0.45	4.5±0.28	NS[c]
	10	40.0±10	22.4±2.0	<0.05
	100	90.2±7.7	56.8±6.5	<0.05
IGF-1	0	0	0	—
	1	23.1±4.8	16.2±0.76	NS
	3	50.9±1.0	38.2±3.7	NS
	10	122.0±1.2	41.3±0.99	<0.05

Stimulation activity was determined by subtracting the amount of basal [^{3}H]thymidine incorporation from that after growth factor stimulation. The numbers represent mean±SD of triplicate assays.

a) Basal levels of [^{3}H]thymidine incorporation into NL-17 and NL-44 were 58.5(±5.5) and 11.3(±1.5) pmol/5×10^4 cell, respectively.

b) Probability of no difference between NL-17 and NL-44 determined by Student's *t* test.

c) Not significant.

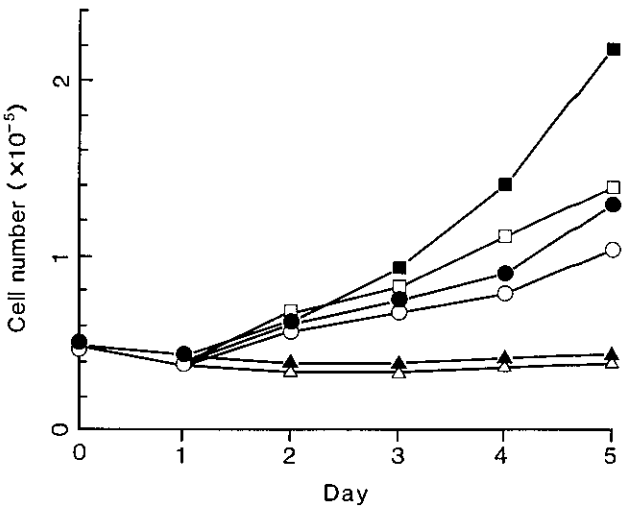

Fig. 2. Cell growth of NL-17 and NL-44 stimulated by insulin. The cells were plated on day 0, and the serum-free culture with or without insulin was started on day 1 as described in "Materials and Methods." The cell numbers represent the average of triplicate assays. Standard deviation was usually less than 18% of the average. NL-17, closed symbols; NL-44, open symbols; 100 ng/ml of insulin, (■, □); 10 ng/ml of insulin (●, ○); without insulin (▲, △).

Binding of insulin and IGF-1 to NL-17 and NL-44 cells

To examine the binding of insulin to NL-17 and NL-44 cells, the cells were incubated with [^{125}I]insulin in KRP buffer containing 0.1% BSA at 4°C for 15–18 h (Table III). H35 rat hepatoma cells were used as a positive control.[9] The amount of [^{125}I]insulin bound to NL-17 and NL-44 cells was unexpectedly low (less than 10% of H35-bound [^{125}I]insulin). Maximal specific binding to 2.8×10^5 NL-17 cells was only 0.9% of total added radioactivity of [^{125}I]insulin. The specific binding of [^{125}I]-insulin to the cells increased slightly when these cells were previoulsy washed with 0.02 *M* sodium acetate buffer containing 0.5 *M* NaCl at pH 5.5, which eliminates pre-bound insulin from the cells.[10] The extremely low levels of cell bound [^{125}I]insulin did not permit us to perform further analysis.

Under the same conditions, a substantial amount of [^{125}I]IGF-1 bound to NL-17 and NL-44 cells (Fig. 4). Twenty-four percent of the total added radioactivity of [^{125}I]IGF-1 bound to NL-17 cells and 18% bound to NL-44 cells. Scatchard analysis revealed that NL-17 and NL-44 had almost equal numbers of IGF-1 binding sites (receptors), which had similar dissociation constant values; NL-17: 1.37×10^5/cell, *Kd* 8.94×10^{-10} *M*; NL-44: 1.26×10^5/cell, *Kd* 9.54×10^{-10} *M*. We examined insulin for competitive binding to the cell surface with [^{125}I]IGF-1 and found that insulin weakly inhibited the

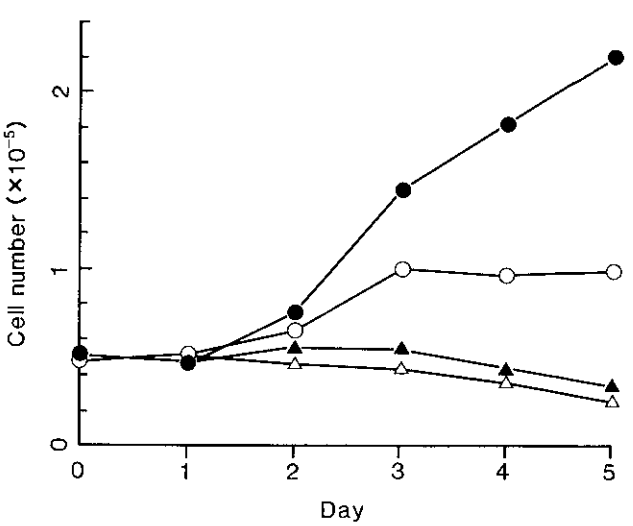

Fig. 3. Cell growth of NL-17 and NL-44 stimulated by IGF-1. The cells were plated on day 0, and the serum-free culture with or without IGF-1 was started on day 1 as described in "Materials and Methods." The cell numbers represent the average of triplicate assays. Standard deviation was usually less than 11% of the average. NL-17, closed symbols; NL-44, open symbols; 10 ng/ml of IGF-1, (●, ○); without IGF-1, (▲, △).

Table III. Binding of [^{125}I]Insulin to NL-17, NL-44 and H35

Cell line	pH of pre-wash	Specific binding of [^{125}I]insulin fmol/10^6 cells[a)]
NL-17	7.4	1.14 ± 0.34[b)]
	5.5	1.25 ± 0.20[c)]
NL-44	7.4	0.35 ± 0.07
	5.5	0.57 ± 0.13
H35	7.4	11.7 ± 2.1
	5.5	7.68 ± 0.49

a) Mean±SD of 3 determinations.
b) Significantly larger ($P<0.05$) than NL-44 prewashed at pH 7.4.
c) Significantly larger ($P<0.05$) than NL-44 prewashed at pH 5.5.

[^{125}I]IGF-1 binding. The EC_{50}value of insulin to compete with [^{125}I]IGF-1 in the binding assay was 260 n*M*, while the EC_{50} value of cold IGF-1 was 1.3 n*M*. There was no significant difference in the EC_{50} values between NL-17 and NL-44 cells. These data suggest that both cell variants have specific receptors with high affinity to IGF-1.

DISCUSSION

The development of metastasis is directly related to the proliferation of tumor cells at the secondary site. The proliferation could be controlled by growth factors supplied at the microenvironment where tumor cells lodge. During the metastatic process, growth factors present in the microvasculature system at the secondary

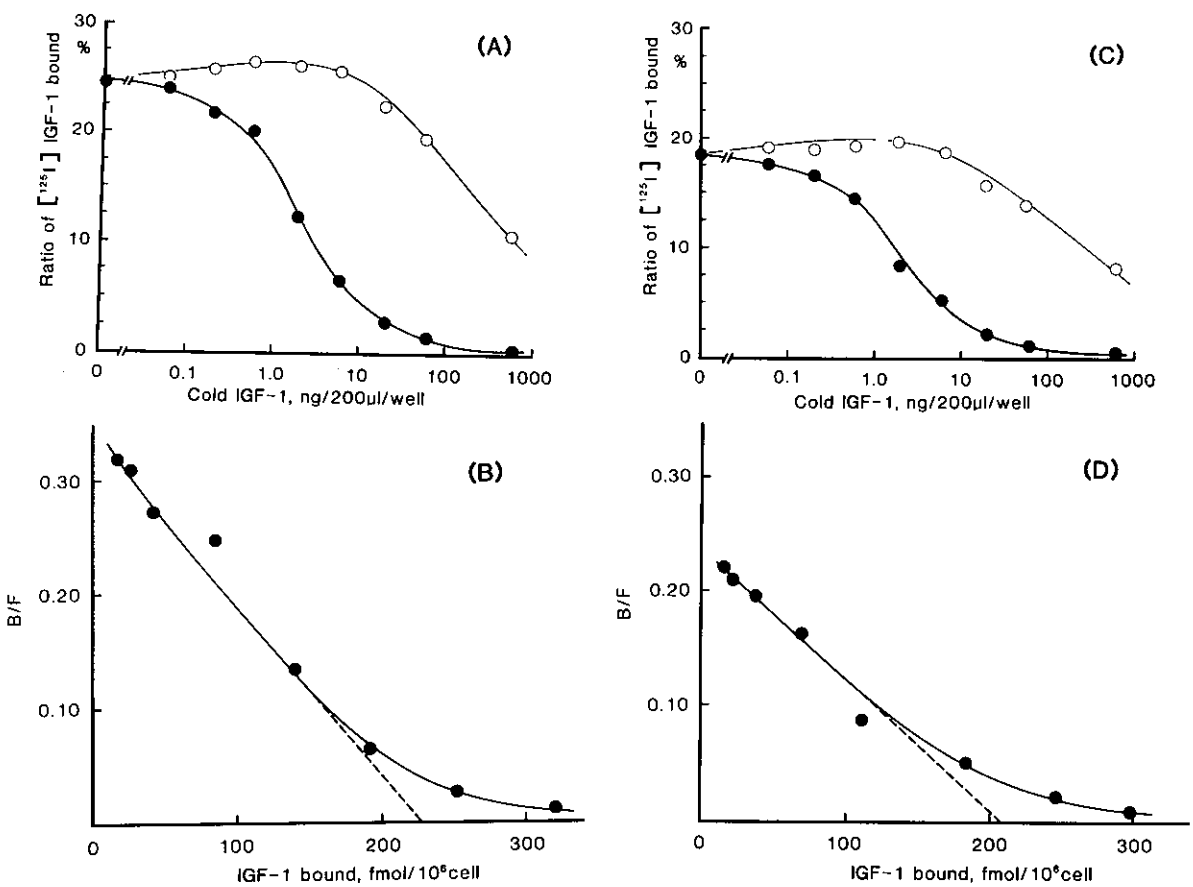

Fig. 4. Competitive binding experiments of [^{125}I]IGF-1 and Scatchard plot analysis. Each point represents the mean value of triplicate assays. Standard deviation was usually less than 8% of the average. (A) Competition of [^{125}I]IGF-1 binding to NL-17 by unlabeled IGF-1 and insulin. (B) Scatchard plot analysis of [^{125}I]IGF-1 binding to NL-17. (C) Competition for [^{125}I] IGF-1 binding to NL-44 by unlabeled IGF-1 and insulin. (D) Scatchard plot analysis of [^{125}I]IGF-1 binding to NL-44. Competition with IGF-1, closed symbols (●); competition with insulin, open symbols (○).

site seem to be important for the development of metastases. To elucidate the significance of growth factors in the establishment of blood-born metastases, we examined their effect on the proliferation of two colon adenocarcinoma 26 variants (NL-17 and NL-44) which have different metastatic potentials. Of the growth factors examined, EGF, TGF$_\beta$, MSA, basic EGF, transferrin, and hydrocortisone did not affect the growth of tumor cells. In contrast, insulin and IGF-1 stimulated the cellular proliferation and DNA synthesis of NL-17 and NL-44 cells, and notably, did so to a significantly greater extent in NL-17 cells (high-metastatic) than in NL-44 cells (low-metastatic). Similarly, Rodeck *et al.* using a chemically defined medium found that proliferation of metastatic melanoma cell lines was stimulated better by insulin and IGF-1 than that of the parental cell line.[12] Insulin not only mediated major physiological responses as a classical hormone but also promoted the proliferation of various cell types by itself[13] and by potentiating the effect of other growth factors.[14] IGF-1 is a growth stimulatory peptide structurally homologous to proinsulin,[15] and is known to be produced primarily by the liver and released into the blood. IGF-1 was more prominent as a growth stimulator of NL-17 cells than insulin; IGF-1 stimulated the NL-17 cell growth at a concentration of 10 ng/ml, which is one-tenth of the effective concentration of insulin and less than the physiological concentration (~200 ng/ml) of IGF-1 reported previously.[16] The mRNA of IGF-1 has been recently demonstrated in the connective tissue of various organs including the lung, suggesting the local production of IGF-1.[17–19] It is therefore conceivable that IGF-1, circulating in the blood or produced and localized in the lung tissue, could stimulate the proliferation of NL-17 cells arrested in the lung.

In order to analyze the mechanism of tumor cell growth stimulation by insulin and IGF-1,[20] we examined the number of insulin and IGF-1 receptors on NL-17 and NL-44 cells. The binding of [^{125}I]insulin to these cells was extremely low compared with the binding to H35 cells, which are known to be stimulated for proliferation through insulin receptors[9] We could not make Scatchard analyses of the [^{125}I]insulin binding to the tumor cells because of the low levels of binding. On the other hand, substantial amounts of [^{125}I]IGF-1 bound to both NL-17 and NL-44 cells. Scatchard analysis unexpectedly revealed that NL-17 and NL-44 had almost equal numbers of IGF-1 receptors, which had similar dissociation constants. Insulin showed weak cross-specificity to the IGF-1 receptor as measured by competition with [^{125}I]IGF-1 for binding to the cell surface. The EC_{50} values of IGF-1 and insulin were 1.3 n*M* and 260 n*M*, respectively. Therefore, it is concluded that the IGF-1 activates the cellular growth through the specific IGF-1 receptor. Compared with IGF-1, ten times more insulin was needed to stimulate the growth of NL-17 and NL-44. This observation is in agreement with the results that the binding of [^{125}I]insulin to the cell surface was ten times lower than that of [^{125}I]IGF-1 when equimolar [^{125}I]insulin or [^{125}I]IGF-1 was added to the tumor cells. Although the numbers and the dissociation constants of IGF-1 receptors were not significantly different between NL-17 and NL-44 cells, the growth of NL-17 cells was more efficiently stimulated by IGF-1 and insulin than that of NL-44 cells. The reason for the difference is unknown. The proliferation responses of NL-17 and NL-44 might be modulated through different postreceptor mechanism(s).[21]

In the above cell growth assay and receptor assay, we must consider the possibility of preoccupation or down-regulation of the cellular receptor. In our experiments, the cells were starved for 8 h in the synthetic medium before binding assay. In the cell growth assay the cells were incubated for 24 h in synthetic medium containing 1% fetal bovine serum to allow the cells to attach to the surface of the dishes. These procedures might not eliminate the possibility of preoccupation or down-regulation of the growth factor receptor. However, we carried out a thymidine incorporation experiment after 2 days of starvation (Table II), and found that the results were similar to those obtained without serum starvation (data not shown). These findings might suggest a negligible or marginal involvement of preoccupation or down-regulation of the cellular receptors of the growth factors in the present experiments.

In addition to circulating growth factors, other growth factors could be considered relevant to the *in vivo* growth of metastatic cells. For example, these include growth-promoting activities localized in host organs[22–24] and growth factors possibly released from host cells at the metastatic site following interaction with tumor cells. Autocrine growth factor(s) of metastatic cells may include another potentiation factor for metastases. There are a number of determinants for tumor cells to establish metastases, e.g., presence of specific enzymes for the degradation of basement membrane and extracellular components,[25, 26] and receptors for adhesive molecules.[27–29] In fact, in the lung colonization of iv-injected colon 26 cells, we demonstrated that platelet aggregation by tumor cells is one of the determinants of metastasis.[4, 6] In addition to these determinants, high sensitivity of tumor cells to circulating growth factors is likely be another determinant which enhances the survival and growth of tumor cells at the secondary sites. At least in our colon 26 lung-colonizing system, insulin and IGF-1 may selectively enhance the survival and growth of the tumor cells in the capillary bed of the lung, resulting in establishment of blood-born metastases.

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