Isolation and Characterization of Invasive and Noninvasive Variants of a Rat Bladder Tumor Cell Line

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We isolated, in vitro, spontaneous variants of the rat bladder tumor NBT-II cell line with a distinctive morphology. Of five sublines obtained, three (NBT-L1, L2a and L2b) exhibited an elongated shape and moderate to high invasive activity in vitro. The other two sublines (NBT-T1 and T2) formed tight colonies and exhibited very low or negligible invasive activity. The contents of mRNAs coding for E-cadherin and cadherin-associated molecules (α-catenin and β-catenin) were not correlated with the invasive activity of the cells. However, the expression level of the E-cadherin protein, but not those of catenins, was lower in invasive cells (NBT-L1, L2a and L2b) than in noninvasive cells (NBT-T1 and T2). Analysis of mRNAs coding for several growth factors and their receptors showed that the transforming growth factor α mRNA content in invasive cells was higher than that in noninvasive cells, and that the content of epidermal growth factor receptor mRNA was low in NBT-T2. Although NBT-II is known to acquire a fibroblastic appearance and cell motility in response to several growth factors, the conditioned media of the invasive sublines hardly affected the morphology or motility of noninvasive cells. These results indicate that the decreased E-cadherin expression is closely associated with the transition from the noninvasive to the invasive phenotype of the bladder tumor cells, and that a post-transcriptional process is important in the control of E-cadherin expression in the cells. These sublines may be useful as models for studies on the progression of bladder tumors.

Key words: Bladder tumor — Invasion — E-cadherin — Catenin — Cell line

Most human urinary bladder tumors are transitional cell carcinomas of noninvasive superficial type and they are easily managed by transurethral manipulation. Superficial bladder tumors, however, very frequently recur, and some of the recurrent tumors progress to the invasive form, resulting in a poor prognosis.

Disturbance of cell adhesion and increased cell motility are key components of the invasive and metastatic process. Although the factors involved in the process are not fully known, there is substantial experimental and clinical evidence that cell adhesion molecules and growth factors are directly involved in the dissociation of and the acquisition of motility by malignant cells, respectively.[1, 2] Among a variety of cell adhesion molecules, E-cadherin has been studied most extensively in relation to the metastatic process. A strong correlation has been reported between decreased E-cadherin expression, and the invasion and metastasis of various tumors, including bladder ones.[3] Conversely, forced expression of E-cadherin in nonexpressing tumor cells has been shown to induce cell contact and to prevent invasion in vitro.[4, 5] In addition to the decreased expression of E-cadherin itself, the deprivation of functional catenins, which mediate the linkage between cadherins and the actin cytoskeleton, and are indispensable for cadherin function, results in

disturbance of cell adhesion and an increase in the invasive ability of tumor cells.[6, 7]

Our previous study showed that the contents of mRNAs for c-met/hepatocyte growth factor (HGF) receptor and transforming growth factor α (TGF-α) increased during bladder carcinogenesis induced by N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), and that HGF, also known as scatter factor, stimulated the growth and motility of a rat bladder tumor cell line (NBT-II).[8] Bellusci et al.[9] reported that HGF can promote the motility and invasive character of NBT-II transfected with an expression vector for human HGF in an autocrine manner. They also found that a metastatic variant of NBT-II produces a scatter factor-like factor which stimulates the dissociation of parental NBT-II cell clusters.[10] In addition to HGF, the results of in vitro studies have suggested that basic fibroblast growth factor (bFGF) and TGF-β1 are involved in the invasion of bladder carcinoma.[11, 12]

In the previous study, we found spontaneous variants of NBT-II. The variants could be classified into two groups according to their morphology: two of them formed round, tight colonies, while the other three exhibited an elongated shape and increased cell motility, indicating reduced cell-cell contact. In addition, the morphology of the cells was correlated with their invasive activity in vitro. Here we report the isolation and characterization of these NBT-II variants. The expression of

E-cadherin, catenins, growth factors and growth factor receptors was examined in relation to the motility and invasive character of the cells.

MATERIALS AND METHODS

Cell culture and isolation of NBT-II variants The NBT-II cell line,[13] obtained from ICN Biomedicals Inc. (Costa Mesa, CA), was routinely grown on 100-mm tissue culture dishes in minimum essential medium with Earle's salts supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10% fetal bovine serum (standard medium). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For the isolation of variants, NBT-II was seeded at a density of 300 cells/100-mm dish. After 6 days of culture, colonies with a distinctive morphology were isolated using a cloning ring. Five stable NBT-II sublines (NBT-T1, T2, L1, L2a and L2b) were obtained after three rounds of colony isolation.

Cell motility assay Trypsinized cells were inoculated into 6-well cell culture plates previously coated with extracellular matrix (ECM) proteins at a density of 3×10^3 cells/well. Fibronectin (Imcera Bioproducts Inc., Terre Haute, IN), laminin (Biomedical Technologies Inc., Stoughton, MA), and type I collagen (Koken, Tokyo) were adsorbed on the plastic by incubating the plates with 1 ml/well of ECM protein solutions, 10 μg/ml in phosphate-buffered saline (PBS), for 24 h at 37°C in a CO_2 incubator. Control wells were treated with PBS. The wells were washed three times with 2 ml of PBS before use. The samples to be assayed were added 6 h after plating of the cells. The cells were cultured 3 days before staining with Giemsa's solution.

In vitro **invasion assay** Cells suspended in 2 ml of the standard medium (1×10^5 cells/ml) were added to a rehydrated Matrigel invasion chamber (Becton Dickinson, Bedford, MA). The chamber was placed in a well of a 6-well culture plate containing 2 ml of standard medium, and then the cells were cultured for 40 h. At the end of the culture period, the Matrigel layer was removed with a cotton swab and the invading cells attached to the underside of the filter were stained with Giemsa's solution. After drying, the filter was immersed in liquid paraffin, making it transparent. A plastic film ruled into 2-mm squares was placed on the underside of the filter, dividing it into 32 sections, and the stained cells were counted under a microscope. The results given in the text represent the averages for six chambers for each subline.

Northern blot analysis Northern blot analysis was carried out as described previously.[14] Briefly, poly(A)$^+$ RNA (1.5 μg) from subconfluent cultures of the NBT-II variants was electrophoresed on a 1% agarose/2.2 M formaldehyde gel, and then transferred to a nylon membrane. After prehybridization, the blots were incubated with probes labeled with digoxigenin-11-dUTP (see below) for 15 h at 65°C. The membrane was washed with $2 \times$ SSC/0.1% SDS at room temperature, and then with $0.1 \times$ SSC/0.1% SDS at 65°C. The hybridized probe was detected on X-ray film after the development of chemiluminescence using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim GmbH, Mannheim, Germany) and Lumi-Phos 530 (Lumigen Inc., South Field, MI). Blots which hybridized to different probes were washed with 0.1% SDS for 30 min in a boiling-water bath. All the probes were labeled with digoxigenin-11-dUTP by the method of Lanzillo,[15] as described previously.[14]

Western blot analysis Western blot analysis was performed as described previously.[16] Briefly, cells were cultured to subconfluency, washed twice with PBS, and then solubilized with a lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 8 M urea; 2 ml/100-mm dish). The cell lysates were scraped into tubes, and then heated at 98°C for 3 min. Samples (15 μg protein/lane) were electrophoretically separated in SDS/10% polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with monoclonal antibodies against E-cadherin, α-catenin, β-catenin, and plakoglobin/γ-catenin (Transduction Laboratories, Lexington, KY). The bound antibodies were detected on X-ray film after the development of chemiluminescence, using Lumi-Phos 530 and an alkaline phosphatase-labeled antibody to mouse IgG (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). The film was scanned at 520 nm with a densitometer (model CS-910; Shimadzu Seisakusho, Kyoto) to determine the relative E-cadherin content. Protein was determined by the method of Lowry *et al.*[17] on duplicate plates.

Immunofluorescence staining of E-cadherin Cells cultured on a slide glass were washed with PBS and then fixed in methanol for 15 min at −20°C. The fixed cells were incubated with an anti-E-cadherin monoclonal antibody (1.25 μg/ml in PBS, Transduction Laboratories) for 16 h at 4°C, following pre-treatment with PBS containing 10% goat serum for 1 h at room temperature. The cells were washed with PBS, incubated with FITC-conjugated goat anti-mouse IgG (50 μg/ml; Kirkegaard & Perry Laboratories Inc.) for 30 min at room temperature, washed with PBS, and then analyzed by confocal laser scanning microscopy.

RESULTS

Cell motility and invasive activity *in vitro* of the NBT-II sublines We have isolated five sublines, designated as NBT-T1, T2, L1, L2a and L2b, of the rat bladder tumor
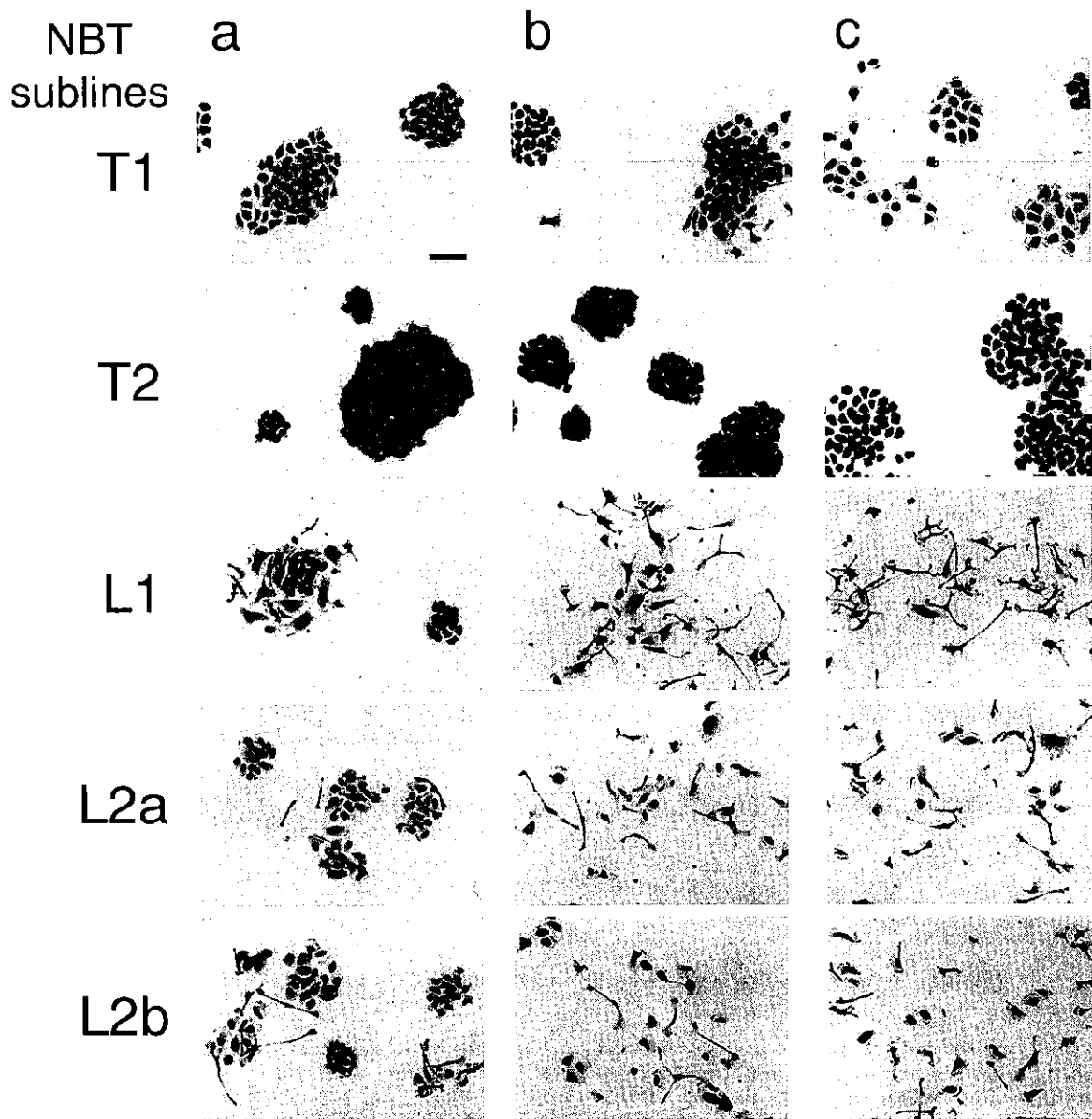
Fig. 1.   Morphology of the NBT-II sublines. Cells cultured on plain tissue culture plate (a), laminin-coated plate (b), and type I collagen-coated plate (c) were stained with Giemsa's solution. Bar, 100 μm.

NBT-II cell line. NBT-T1 and T2 (T-series sublines) formed round, tight colonies on plastic tissue culture plates, while NBT-L1, L2a and L2b (L-series sublines) exhibited an elongated shape and formed loose colonies, indicating that cell-cell adhesiveness is reduced in these sublines (Fig. 1). All the sublines grew rapidly under the standard culture conditions, the average doubling times being 13.4 h, 12.8 h, 16.8 h, 15.6 h and 14.2 h for NBT-T1, T2, L1, L2a and L2b, respectively. The differences in cell morphology and motility between the T- and L-series

sublines became more prominent when they were cultured on plates coated with ECM proteins (Fig. 1). The L-series cells could not form distinct colonies on laminin-coated plates and dispersed into single cells almost completely on type I collagen-coated plates. Although the cell-cell contact of NBT-T1 and T2 was also weak on type I collagen, they maintained a round cell shape, which was different from the highly elongated shape of L-series cells. NBT-T2 was least susceptible to ECM protein-induced cell dispersion. The effects of fibronectin
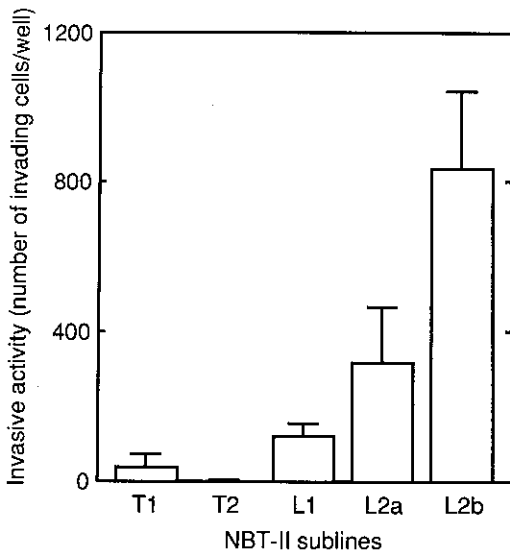
Fig. 2. *In vitro* invasive activity of the NBT-II sublines. Cells (2×10^5) were cultured in a Matrigel invasion chamber for 40 h. At the end of the culture period, the invading cells attached to the underside of the filter were stained and counted under a microscope. Vertical bars represent the means±SEM for six samples.
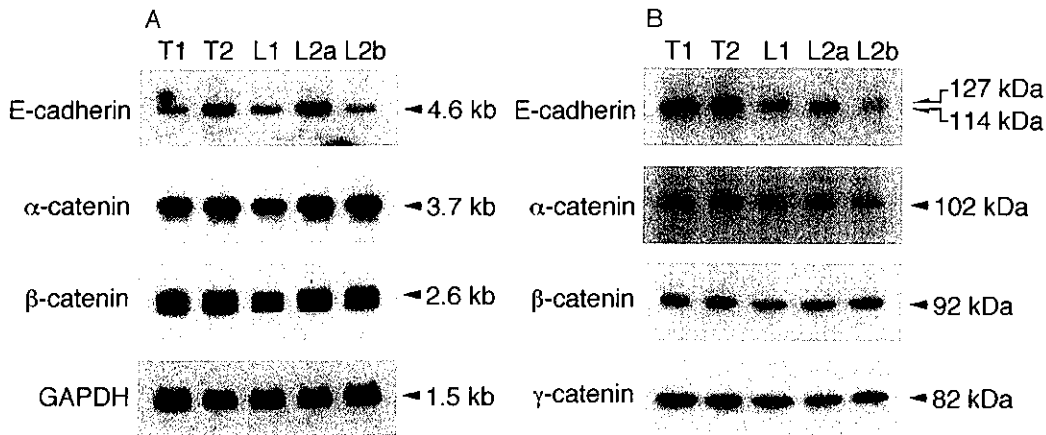

Fig. 3. Northern blot and western blot analyses of E-cadherin and catenins in the NBT-II sublines. A, Northern blot analysis of E-cadherin, α-catenin and β-catenin mRNAs. Poly(A)$^+$ RNA (1.5 μg) from subconfluent cultures of the NBT-II sublines was used. Blots were successively hybridized to cDNA probes for E-cadherin, α-catenin, β-catenin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the hybridized probes were detected on X-ray film after chemiluminescence development. B, Western blot analysis of E-cadherin, α-catenin, β-catenin and γ-catenin. Cell lysates (15 μg protein/lane) were electrophoretically separated in SDS/10% polyacrylamide gels and then transferred to PVDF membranes. The membranes were incubated with monoclonal antibodies against E-cadherin, α-catenin, β-catenin and γ-catenin. The bound antibodies were detected on X-ray film after chemiluminescence development using Lumi-Phos 530 and an alkaline phosphatase-labeled antibody to mouse IgG.

and type IV collagen on the cells were similar to that of laminin (data not shown).

To determine whether or not the cell motility was correlated with the invasive potential of the sublines *in vitro*, we utilized the Matrigel invasion chamber assay. NBT-T2 could hardly invade the Matrigel layer during culture for 40 h, and NBT-T1 showed low invasive

activity. The invasive activities of NBT-L1, L2a and L2b were about 3, 9 and 23 times, respectively, that of NBT-T1 under the conditions used (Fig. 2).

Expression of E-cadherin and catenins On northern blot analysis, the expression of mRNAs for proteins involved in E-cadherin-mediated cell-cell adhesion (E-cadherin, α-catenin and β-catenin) was detected in all the sublines

(Fig. 3a). There was an apparent difference in the expression level of E-cadherin mRNA among the sublines, but it was not correlated with the invasive activity *in vitro*. On the other hand, western blot analysis of E-cadherin and catenins showed that E-cadherin was decreased in the L-series sublines (Fig. 3b). The relative E-cadherin contents were 100, 117, 24, 19 and 7 for NBT-T1, T2, L1, L2a and L2b, respectively. This was consistent with the results of immunocytochemical analysis of E-cadherin. In NBT-T1 and T2 cells, E-cadherin was clearly observed at areas of cell-cell contact, while it was reduced in NBT-L1 and L2b cells, being diffusely distributed in the cytosol (Fig. 4). Western blot analysis also revealed the presence of two kinds of E-cadherin molecule with apparent molecular weights of about 127,000 and 114,000. The latter molecular species was clearly detected only in L-series sublines (Fig. 3b). In contrast to E-cadherin, the contents of catenins, including plakoglobin/γ-catenin, in L-series cells were comparable to those in T-series cells (Fig. 3b).

Expression of growth factors and their receptors The expression of mRNAs for several growth factors and their receptors, which may affect the cell motility and invasiveness, was examined in the NBT sublines. The content of TGF-α mRNA was high in L-series cells, and those of EGF receptor mRNA and TGF-β1 mRNA were low in NBT-T2 (Fig. 5). The expression of mRNAs for HGF, EGF and bFGF was not detected in any of the sublines. The mRNAs for TGF-β type II receptor, c-met/HGF receptor and FGF receptor 1 were expressed

in all the sublines, but they exhibited no apparent relation to the invasive activity of the sublines (Fig. 5).

Effects of growth factors and conditioned media of invasive cells on the motility of NBT-T1 and T2 NBT-T1 and T2 responded to the cell-scattering action of HGF in a similar manner to NBT-II (data not shown). TGF-α also stimulated the cell motility of these sublines, but its
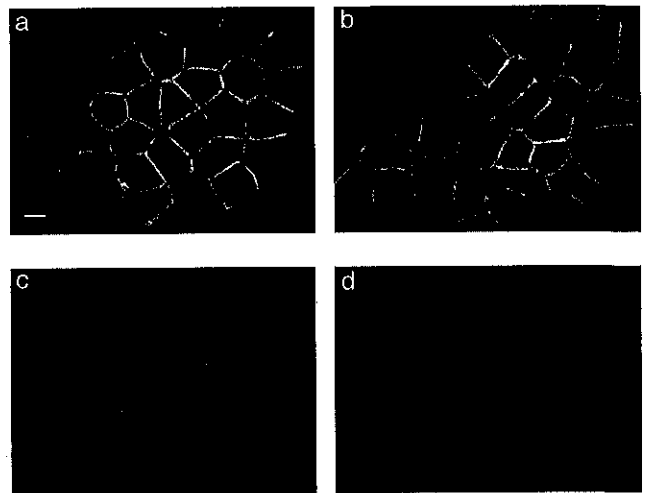

Fig. 4. Immunofluorescent staining of E-cadherin in the NBT-II sublines. a, NBT-T1; b, NBT-T2; c, NBT-L1; d, NBT-L2b. Bar, 10 μm.
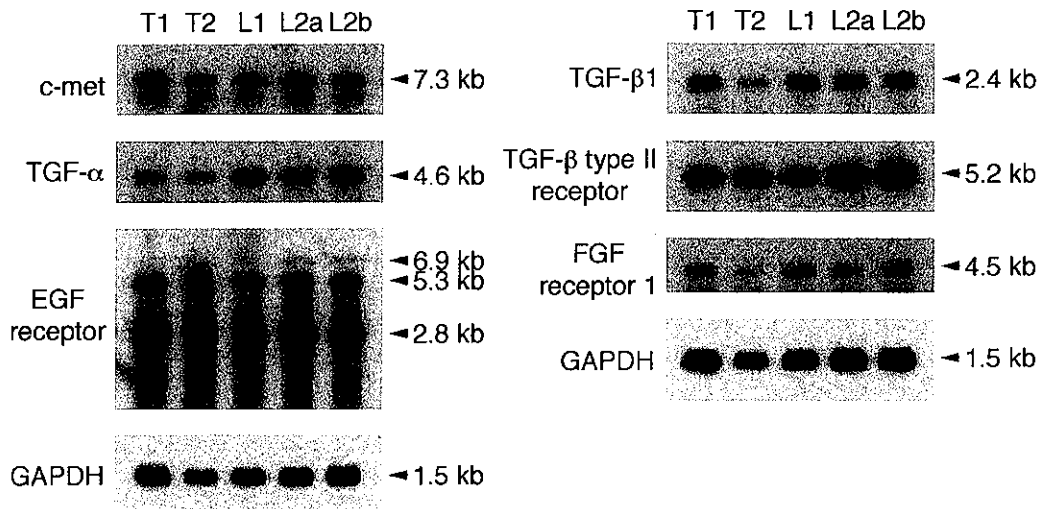

Fig. 5. Northern blot analysis of mRNAs for growth factors and growth factor receptors in the NBT-II sublines. Blots were successively hybridized to cDNA probes for c-met, TGF-α, EGF receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or TGF-β1, TGF-β type II receptor, FGF receptor 1 and GAPDH. The other conditions were as given in the legend to Fig. 3.
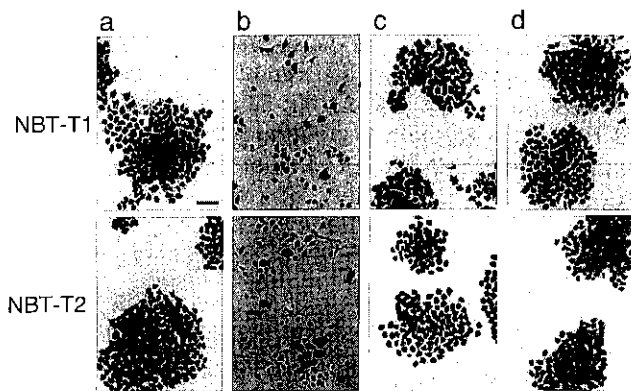
Fig. 6. Effects of TGF-α, and conditioned media of NBT-L1 and -L2b on the motility of NBT-T1 and T2 cultured on type I collagen-coated plates. Cells were cultured in the standard medium (a), or in the standard medium containing TGF-α (3 ng/ml) (b), 50% (v/v) NBT-L1 conditioned medium (c), 50% (v/v) NBT-L2b conditioned medium (d) for 3 days before staining with Giemsa's solution. Bar, 100 μm.

action was weaker than that of HGF. The cell-scattering action of TGF-α was prominent when the cells were cultured on type I collagen-coated plates (Fig. 6). On the other hand, conditioned media of NBT-L1 and L2b, when added at concentrations of up to 50% (v/v), hardly affected the motility of NBT-T1 and T2 cultured on type I collagen-coated plates (Fig. 6). Conditioned medium of NBT-L2a was also ineffective (data not shown).

DISCUSSION

In this study, we isolated five sublines of the rat bladder tumor NBT-II cell line obtained from a commercial source. The parental cell line was not homogeneous as to cell morphology, and was composed of mainly NBT-T1-like cells and less abundant NBT-T2-like cells. Although the morphology of NBT-T1 is similar to that of NBT-II used in other laboratories, we can not conclude that NBT-T1 is identical to the original NBT-II isolated by Toyoshima et al.[13] Accordingly, we used the designations, NBT-T1, T2, L1, L2a and L2b, throughout the present study.

The L-series cells, probably derived from NBT-T1 through spontaneous transformation, showed reduced cell-cell adhesiveness and higher invasive activity in the invasion chamber assay, in contrast to the firm cell-cell attachment and low to negligible invasive activity of the T-series cells. Among the factors examined, E-cadherin showed the highest correlation with the in vitro invasive activity. Although E-cadherin mRNA was expressed in

all the sublines and the content of the mRNA showed no correlation with the invasive activity, the content of the E-cadherin protein was greatly reduced in the L-series cells (Fig. 3b). Cadherins are anchored to the cytoskeletal actin filament via cytoplasmic proteins called catenins (α-, β- and γ-catenins). This anchoring is indispensable for the function of E-cadherin. A lack of catenin expression[6, 7] and increased phosphorylation of β-catenin[18] have been reported to induce the disruption of E-cadherin-mediated cell-cell attachment, even if a normal E-cadherin level is maintained. We could not detect any differences in the expression of catenins at either the mRNA or protein level among the sublines. Although we have not examined the phosphorylation of β-catenin, these results indicate that down-regulated E-cadherin expression is probably an immediate cause of the increased invasive activity of the L-series cells.

There is a good correlation between E-cadherin immunohistochemistry, and the mRNA levels in various cell lines and human cancer specimens,[3] that is, E-cadherin expression is transcriptionally regulated in most cases. As described above, such a correlation was not observed in the NBT-II sublines. The discrepancy between the E-cadherin mRNA and protein contents in these sublines suggests that a post-transcriptional process is important in the regulation of E-cadherin expression in bladder tumor cells. In addition to the decreased E-cadherin contents of the L-series cells, western blot analysis revealed that the NBT-II sublines contained two types of E-cadherin molecules (Fig. 3b). A molecular species with an apparent molecular weight of about 127,000 was common to all the sublines, while one with a slightly faster electrophoretic mobility (M.W. = ca. 114,000) was clearly detected only in the L-series cells. Mutations of the E-cadherin gene have been found in human gastric cancer.[19] The mutation hot spots are located at splice sites, leading to exon skipping. Although the presence of a mutant E-cadherin molecule was not investigated in gastric cancer, Matsuura et al.[20] reported that E-cadherin in cancer cells from pleural effusion of lung adenocarcinoma had a molecular weight slightly lower than that of the intact molecule. If a mutation(s) of the E-cadherin gene generated the smaller E-cadherin molecule in L-series cells, each L-series cell expressed wild and mutant E-cadherins in both alleles or every L-series subline was composed of two types of cells expressing wild or mutant molecule. Another possibility is that the smaller molecule was a degradation product of a mutant E-cadherin protein, which was more susceptible to proteolysis than the wild-type protein. Analysis of the E-cadherin gene of L-series cells is needed to clarify the origin of the smaller E-cadherin molecule.

It has been established that growth factors can enhance the invasive and metastatic abilities of a variety of

tumor cells. For instance, the invasive potential of NBT-II is increased by TGF-α,[21] acidic FGF[22] and HGF[9] in a paracrine and/or autocrine manner. Moreover, a spontaneous metastatic variant of NBT-II was found to produce and secrete a scatter factor-like factor.[10] These observations, together with our previous finding that the expression of c-*met*/HGF receptor and TGF-α mRNAs increased during BBN-induced bladder carcinogenesis, suggest the close association of bladder tumor progression with enhanced expression of growth factor systems, especially the HGF-c-*met* and TGF-α-EGF receptor systems. Northern blot analysis revealed that c-*met* mRNA was expressed in all the NBT-II sublines, but there was no indication of up-regulation of the c-*met*-HGF system in the L-series cells. On the other hand, the content of TGF-α mRNA was increased in the L-series cells, and that of EGF receptor mRNA was decreased in NBT-T2 (Fig. 5). However, it is not probable that overproduction of TGF-α is a cause of the increased invasive activity of the L-series cells, as the T-series cells retained responsiveness to TGF-α, and the conditioned media of the L-series cells had a negligible effect on the morphology and motility of the T-series cells (Fig. 6).

A wide variety of factors, including cell adhesion molecules, growth factors and their receptors, ECM proteins, proteinases and proteinase inhibitors, have been shown to be involved in tumor cell invasion and metastasis. The results obtained in this study indicate that decreased E-cadherin expression is closely associated with the increased invasive activity of the L-series cells. We can not, however, exclude the possibility that factors other than the cell adhesion molecule participate in the malignant transformation of NBT-II. These NBT-II sublines, having a wide range of invasive activities, may be useful as tools for studies on the molecular mechanisms underlying the progression of bladder tumors.

ACKNOWLEDGMENTS

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