THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 2, Issue of January 14, pp. 1276-1283, 1994
Printed in U.S.A.

Suppressed Collagen Gene Expression and Induction of α2β1 Integrin-type Collagen Receptor in Tumorigenic Derivatives of Human Osteogenic Sarcoma (HOS) Cell Line*

(Received for publication, June 1, 1993, and in revised form, August 18, 1993)

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Cell-matrix interactions and integrin-type cell adhesion receptors are involved in the regulation of tumor cell invasion and metastasis. We have analyzed the expression of matrix proteins and their cellular receptors in human osteosarcoma cells (HOS) and in their virally (KHOS-NP) and chemically (HOS-MNNG) transformed tumorigenic subclones. Transformation decreased dramatically the cellular mRNA levels of α1(I) collagen. Concomitantly with down-regulation of collagen mRNA levels the synthesis of the collagen receptor, α2β1 integrin, was induced. No α2 integrin mRNA was found in HOS cells, suggesting that its expression was regulated most probably at the transcriptional level. 5-Azacytidine alone or combined with α2 integrin-stimulating cytokines, transforming growth factor-β1, and interleukin-1β, did not turn on the α2 integrin gene. In chemically transformed cells, however, α2 integrin expression could be regulated by cytokines. Thus, we suggest that HOS cells have a strong element, probably other than cell culture-generated *de novo* promoter methylation, suppressing α2 integrin expression and that this factor is lost in both chemical and viral transformation. Furthermore, the mechanism used by cytokines and malignant transformation to increase α2 integrin expression seems not to be identical. Other transformation-related changes in β1 integrins were (i) reduction of the intracellular pool of precursor β1 (in HOS-MNNG cells), leading to faster maturation rate of β1 subunit and slower maturation rate of α subunits, and (ii) decreased electrophoretic mobility of both α and β1 subunits. At the cellular level both chemical and viral transformation increased cell adhesion to type I collagen.

The ability to interact with extracellular matrix and basement membranes is essential for the malignant cancer cell phenotype (Ruoslahti 1992). Previous studies have indicated altered expression and function of cell adhesion receptors after malignant transformation. The changes detected in transformed cells include both impaired function of receptors supporting the normal cell phenotype and activation of receptors needed in invasion and metastasis.

Integrins are a family of heterodimeric cell adhesion receptors (for review, see Hemler (1990), Ruoslahti (1991), and Hynes (1992)). β1 integrin can form a complex with at least nine different α subunits, namely α1–α8 and αv. The ligand for α8β1 is unknown but all other heterodimers are receptors for extracellular matrix molecules. α5β1 is specific for the arginine-glysine-aspartic acid (RGD) sequence in fibronectin molecule. α6β1 and α7β1 are specific for laminin, but all other heterodimers recognize more than one ligand molecule. α1β1 and α2β1 are receptors for laminin and various collagen types. α3β1 can bind to the same ligands as α1β1 and α2β1 and, in addition, also to the RGD sequence in fibronectin. α4β1 is a lymphocyte-homing receptor and a receptor for fibronectin, recognizing, however, a different domain than the other fibronectin-binding integrins. αvβ1 is a receptor for fibronectin and vitronectin. Thus, one ligand molecule can bind to several integrin heterodimers. Furthermore, one cell can express simultaneously several receptors recognizing the same ligand. The cellular response to the matrix might be dependent on which one of the receptors is used (Chan *et al.*, 1992).

The disappearance of fibronectin from cell surfaces is often seen after malignant transformation (Ruoslahti, 1992). This phenomenon has been explained in some cases by decreased expression of α5β1 integrin (Plantefaber and Hynes, 1989). Furthermore, Giancotti and Ruoslahti (1990) have shown that the overexpression of α5 in transformed cells makes them again to behave like their nontransformed counterparts. However, in many other transformation models α5 integrin expression is not decreased (Akiyama *et al.*, 1990). The fact that integrin localization is disorganized in these cells suggests that transformation can also regulate the function of integrins (Akiyama *et al.*, 1990). Furthermore, in transformed cells glycosylation of integrin molecules can be altered (Van de Water *et al.*, 1988; Symington *et al.*, 1989). Glycosylation has been shown to affect the ligand-binding ability of integrins (Öz *et al.*, 1989). Transformation can also increase the expression of laminin and collagen-binding integrin heterodimers (Dedhar and Saulnier, 1990). A recent report studying rhabdomyosarcoma cells has indicated the essential role of α2β1 collagen receptor in metastasis formation (Chan *et al.*, 1991).

The importance of integrins in cancer biology was also suggested by the observation that synthetic RGD-peptides can prevent the formation of lung metastasis in mice when injected with melanoma cells (Humphries *et al.*, 1986). The same peptide can *in vitro* prevent tumor cell migration (Gehlsen *et al.*, 1988). Furthermore, β1 integrins can also transduct signals into the cells and regulate cell behavior. This has been shown, for example, in experiments where ligand-integrin interaction induced collagenase and stromelysin synthesis (Werb *et al.*, 1989). The regulation of proteinase synthesis by matrix receptors might have importance in cancer cell invasion through the basement membranes.

Most mesenchymal cell lines produce fibronectin and type I collagen in cell culture. Malignant transformation can alter

* This study was supported by grants from the Finnish Cancer Association, the Sigrid Juselius Foundation, and the Academy of Finland. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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also this cellular function. For example, activation of several oncogenes may lead to decreased collagen production. These genes include v-*src*, c-*myc*, v-*mos*, v-*fos*, N-*ras*, and Ha-*ras* (see Slack *et al.* (1992) and references therein).

We have analyzed the changes in type I collagen and fibronectin gene expression and in integrin biosynthesis and maturation in human osteogenic sarcoma (HOS)[1] cell clones transformed with Kirsten sarcoma virus and compared them to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) transformed cells. We report that not depending on the mechanism used to transform the cells, their collagen gene expression is downregulated in the tumorigenic subclones. Concomitantly, the ability of cells to attach to collagen increases and the expression of $\alpha 2\beta 1$ collagen receptor is induced.

MATERIALS AND METHODS

Antibodies—Polyclonal rabbit antiserum was raised against human placental fibronectin receptor as described earlier (Heino *et al.*, 1989). This antiserum recognizes epitopes in the $\beta 1$ integrin subunit but not in the α subunits (Heino *et al.*, 1989). Anti-$\alpha 2$ and anti-$\alpha 3$ integrin subunit antisera were raised in rabbits by immunization with synthetic peptides having the amino acid sequences corresponding to the full-length intracellular domains of $\alpha 2$ and $\alpha 3$ subunits: H_2N-KLGFFKRKYEKMTKNPDEIDETTELSS-COOH ($\alpha 2$ integrin subunit; EMBL/GenBank™ accession no. X17033) and H_2N-KRARTRALYEAKRQKAEMKSQPSETERLTDDY-COOH ($\alpha 3$ integrin subunit; EMBL/*GenBank*™ accession no. M59911). Peptides were linked to keyhole limpet hemocyanin (Calbiochem) as described by Staros *et al.* (1986), mixed with Freund's complete adjuvant, and injected subcutaneously into rabbits. After three boosters, immunization was continued with free peptides mixed with Freund's incomplete adjuvant. Antisera were compared to previously described monoclonal antibodies, 12F1 (Pischel *et al.*, 1987), and J143 (Kantor *et al.*, 1987), specific for $\alpha 2$ and $\alpha 3$ subunits, respectively. Expression of $\alpha 1$, $\alpha 5$, and $\alpha 6$ integrin subunits was tested with the specific monoclonal antibodies TS2/7 (Hemler *et al.*, 1984), BIIG2 (Werb *et al.*, 1989), and G0H3 (Sonnenberg *et al.*, 1988), respectively.

Cell Cultures—The following human osteosarcoma cell lines were used: MG-63, HOS, HOS-MNNG (HOS cells transformed with MNNG, tumorigenic), KHOS-NP (Kirsten murine sarcoma virus transformed, tumorigenic) and KHOS-240 (Kirsten murine sarcoma virus transformed, non-tumorigenic) all from American Type Culture Collection. To test the new antisera, we used human squamous cell carcinoma (SCC) cells, human gingival fibroblasts, rat skin fibroblasts, bovine skin fibroblasts, and green monkey kidney (GMK) cells cultured in Dulbecco's modification of Eagle's medium (DMEM; Flow Laboratories, Irvine, United Kingdom) supplemented with 10% fetal calf serum (FCS; Flow Laboratories), and L6E9 rat myoblasts (Nadal-Ginard, 1978) grown in DMEM supplemented with 20% FCS (Flow Laboratories).

Cytokines and Growth Factors—Purified bovine bone transforming growth factor-$\beta 1$ (TGF-$\beta 1$) was kindly provided by Dr. J. Massagué (Sloan-Kettering Institute, New York). Human recombinant interleukin-1β (IL-1β) was purchased from Boehringer Mannheim. One milligram of IL-1β is equal to more than 10^7 units in the mouse thymocyte assay.

In the experiments studying the effects of cytokines, cells were incubated with them overnight in serum-free DMEM before metabolic labeling and immunoprecipitation assays. The concentration used was 200 pM for TGF-$\beta 1$ and 10 units/ml for IL-1β. In one set of experiments cells in 5% FCS containing DMEM were exposed for 24 h to 10 μM 5-azacytidine (Sigma) alone or in combination with cytokines.

Immunoprecipitations—Cells were labeled with 50 or 100 μCi/ml [^{35}S]methionine (Tran^{35}S-label, ICN Biochemicals) in methionine-free minimum essential medium. Cell monolayers were washed on ice with a solution containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 25 mM Tris-HCl (pH 7.4), followed by detachment of cells by scraping. Cell pellets obtained by centrifugation at 500 × *g* for 5 min were solubilized in 200 μl of the same buffer containing 100 mM *n*-octyl β-D-glycopyranoside (Sigma) on ice with occasional vortexing. The insoluble material was removed by centrifugation at 10,000 × *g* for 5 min at 4 °C. Radioactivity in cell lysates was counted, and a standard amount of radioactivity was used in immunoprecipitation assays. Cytokines did not cause any systematic changes in the incorporation of [^{35}S]methionine into cellular proteins. Triton X-100 (0.05% v/v) and bovine serum albumin (0.5 mg/ml) were added to the supernatants, which were then precleaned by incubation with 50 μl of packed protein A-Sepharose (Pharmacia LKB Biotechnology Inc.). The resulting supernatants were immunoprecipitated with anti-integrin antibodies for 12 h at 4 °C. Immune complexes were recovered by binding to protein A-Sepharose and washing the beads four times with 25 mM Tris-buffered isotonic saline (pH 7.4) containing 0.5% Triton X-100 and 1 mg/ml bovine albumin and twice with 0.5 M NaCl and 25 mM Tris-HCl (pH 7.4). The immunoprecipitates were analyzed by electrophoresis on sodium dodecyl sulfate-containing 6% or 7% polyacrylamide gels followed by fluorography. Integrin bands were quantified from fluorograms by the Microcomputer Imaging Device version M4 (Imaging Research Inc.).

Pulse Chase Experiments—Confluent cultures were preincubated in methionine-free minimum essential medium for 3 h. After preincubation cells were labeled with 100 μCi/ml [^{35}S]methionine in the same methionine-free medium for 10–60 min. Cultures were then shifted to fresh DMEM for the indicated lengths of time. Soluble cell extracts prepared from these cultures were immunoprecipitated and analyzed by gel electrophoresis and fluorography.

Northern Blot Hybridizations—Total cellular RNA was isolated by using guanidine thiocyanate/CsCl method (Chirgwin *et al.*, 1979). RNAs were separated in formaldehyde-containing agarose gels, transferred to nylon membranes (GeneScreen Plus), and hybridized with ^{32}P-labeled (Amersham Corp.) cDNA probes. cDNAs for human $\alpha 2$ integrin, $\alpha 1$(I) collagen, and fibronectin were kindly provided by Dr. M. Hemler (Takada and Hemler, 1989), Dr. E. Vuorio (Mäkelä *et al.*, 1988), and Dr. S. Hakomori (Sekiguchi *et al.*, 1986), respectively.

Cell Adhesion Assays—Coating of a 96-well immunoplate (Maxi Sorp, Nunc, Denmark) was done by exposure to 0.2 ml of phosphate-buffered saline (pH 7.4) containing 4–5 μg/cm^2 laminin (purified from basement membranes of the Engelbreth-Holm-Swarm mouse tumor, Collaborative Reseach), fibronectin (human fibronectin purified from plasma, Boehringer Mannheim), or collagen (type I collagen from lathyric rat skin, Boehringer Mannheim) for 12 h at 4 °C. Bovine serum albumin (1 mg/ml, Sigma)-coated wells were used to measure the unspecific binding. Residual protein absorption sites on all wells were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h at 37 °C. Confluent cell cultures were detached by 0.01% trypsin and 0.02% EDTA. Trypsin activity was inhibited by washing the cells with 1 mg/ml soybean trypsin inhibitor (Sigma). Cells were suspended in minimal essential media (Life Technologies, Inc., 041-02360, containing 264 mg/liter $CaCl_2 \cdot H_2O$ and 200 mg/liter $MgSO_4 \cdot 7H_2O$), or in S-minimal essential media (Life Technologies, Inc., 041-01385, calcium-free, 200 mg/liter $MgSO_4 \cdot 7H_2O$). 10,000 cells were transferred into a well and incubated for 45 min at 37 °C. Non-adherent cells were removed by flushing the wells with medium. Adherent cells were fixed with 2% paraformaldehyde, stained with 5% crystal violet, and washed with distilled water. The immunoplates were allowed to air-dry, and the stained cells were dissolved into 10% acetic acid and spectrophotometrically measured at 600 nm with Multiskan Plus (Labsystems).

The statistical significance of differences in cell adhesion of different cell lines was assessed with one way analysis of variance. Post hoc study of differences was done using Dunnett's multiple comparison method taking the HOS cells as the control group. *p* values less than 0.05 were considered as statistically significant. Statistical computing was performed with SAS statistical program package (SAS Institute, Cary, NC).

RESULTS

Both Chemical and Viral Transformation Induce the Expression of $\alpha 2\beta 1$ Integrin—Type I collagen is the major protein component of the bone matrix. The aim of this study was to analyze the putative changes in the cell-type I collagen interaction after malignant transformation of osteogenic cells. To this purpose, two novel rabbit antisera against putative collagen binding integrins were prepared. Antisera were raised against synthetic peptides corresponding to intracellular sequences of $\alpha 2$ and $\alpha 3$ integrin subunits (Fig. 1*A*). Their specificity was tested in immunoprecipitation assays. Anti-$\alpha 2$ peptide antiserum precipitated a band with an approximated molecular mass of 140 kDa (Fig. 1*B*, 2*A*). Two major bands (140 and 150 kDa) were seen in samples from MG-63 human osteo-

[1] The abbreviations used are: HOS, human osteogenic sarcoma cells; FCS, fetal calf serum; IL, interleukin; TGF, transforming growth factor; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; SCC, squamous cell carcinoma; GMK, green monkey kidney; DMEM, Dulbecco's modified Eagle's medium.

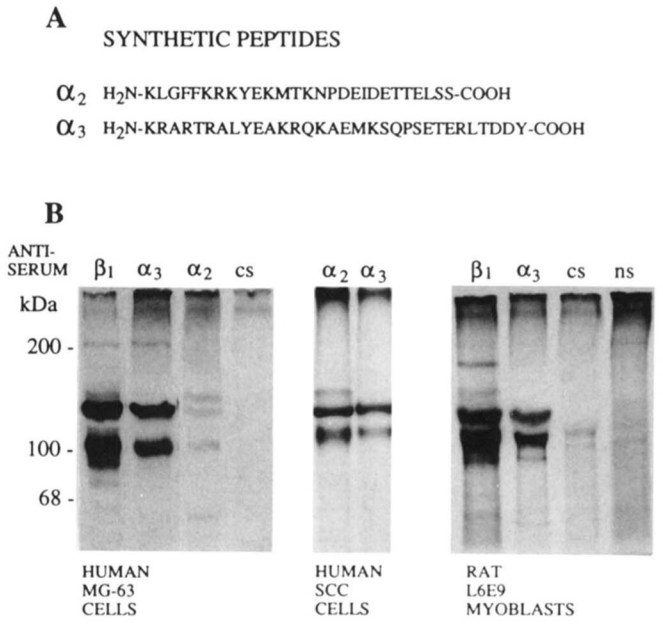

FIG. 1. **Characterization of antisera against synthetic peptides corresponding to COOH-terminal sequences in α2 and α3 integrin subunits.** *A*. synthetic peptides used to immunize rabbits; *B*, immunoprecipitation analyzes with rabbit antisera. MG-63 human osteosarcoma cells, human SCC cells, and rat L6E9 myoblasts were labeled for 20 h with [^{35}S]methionine. Cell membranes were extracted into octylglycoside buffer, incubated with antisera, and the formed immune complexes were harvested with protein A-Sepharose. Precipitated radiolabeled proteins were analyzed by polyacrylamide gel electrophoresis and fluorography. Control precipitations were done without serum (*ns*) or with non-immune rabbit serum (*cs*).

sarcoma cells (Fig. 1*B*), human squamous cell carcinoma (SCC) cells (Fig. 1*B*), and from human gingival fibroblasts (not shown). Identical results were also seen with monoclonal anti-α2 antibody 12F1 (not shown here; for the 12F1 precipitation pattern, see Heino and Massagué (1989)). The identity of the larger α2-related band is not clear. After a 10-min [^{35}S]methionine pulse only the 140-kDa form was found in MG-63 cells. In a pulse chase experiment, the 150-kDa form appears 120 min after the pulse but starts to disappear again after a 150-min chase (not shown). Thus, it is possible that the 150-kDa band represents an intermediate form in the maturation process of α2 integrin. However, we can not exclude the possibility that they are two distinct proteins forming a short-time complex during α2 maturation. Anti-α3 integrin antiserum recognized a protein of about 140 kDa (Fig. 1*B*). This band comigrated with α3 integrins precipitated with specific monoclonal antibodies (not shown). Both anti-α2 and anti-α3 antisera precipitated also a band of about 130 kDa, recognized as the coprecipitating integrin β1 subunit (Fig. 1*B*). We screened several cell lines with previously known integrin patterns to confirm the specificity of these antisera. Anti-α2 antiserum was specific to human and monkey (GMK cell) proteins, whereas the anti-α3 serum was able to immunoprecipitate α3 integrin subunits also from rat (L6E9 myoblasts, skin fibroblasts), and bovine (skin fibroblasts) cell lines (Fig. 1*B*).

We analyzed integrin expression in four human osteosarcoma cell lines. HOS cells are originally cultured from an osteosarcoma but the cell line is not tumorigenic. HOS-MNNG cell line is its chemically transformed, tumorigenic variant (Rhim *et al.*, 1977). KHOS-NP and KHOS-240 cell lines represent Kirsten sarcoma virus-transformed HOS cells (Rhim *et al.*, 1977). KHOS-NP is a tumorigenic and KHOS-240 a non-tumorigenic subclone. Immunoprecipitation of octylglycoside-extracted proteins from metabolically labeled HOS cells with

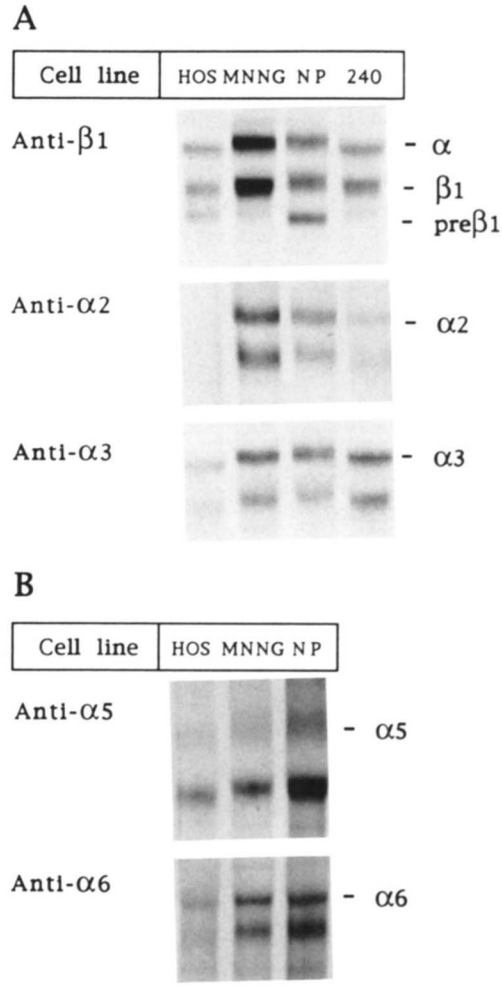

FIG. 2. **Integrin expression in transformed and nontransformed HOS cells.** Detergent extracts of [^{35}S]methionine labeled human osteosarcoma cells were immunoprecipitated with antisera against α2, α3, and β1 (*A*) or α5 and α6 (*B*) integrin subunits. Immunoprecipitated proteins were analyzed by gel electrophoresis and fluorography.

anti-β1 integrin antiserum revealed three major bands (Fig. 2*A*). Based on experiments with other specific anti-integrin antibodies, pulse chase assays, and the use of endoglycosidase enzymes, the bands represent multiple comigrating 140-kDa α integrin subunits, β1 integrin subunit (130 kDa) and intracellular precursor for β1 subunit (110 kDa) (Heino *et al.*, 1989) (experiments shown in this report). Also a fourth, weaker band of about 190 kDa was constantly detected (not shown in Fig. 2*A*, but can be seen in Fig. 4*B*). This was found to represent α1 integrin subunit. Immunoprecipitations with α chain-specific antisera and monoclonal antibodies showed that, in complex with β1, HOS cells expressed α1 (Fig. 4*B*), α3 (Fig. 2*A*), α5, and α6 (Fig. 2*B*) subunits, whereas no α2 integrin expression was seen (Fig. 2*A*). The major observation was that both viral and chemical transformation induced α2 integrin expression (Fig. 2*A*, Table I). To quantify the changes in α2 and α3 integrin expression, we labeled five parallel cell cultures, made the immunoprecipitations and processed the fluorograms with an image analyzer. The results presented in Table I are in agreement with other independent experiments done with fewer parallel samples. The expression level of α2 subunit was constantly lower in 240 cells than in KHOS-NP or HOS-MNNG cells (Fig. 2*A*, Table I). In different experiments, the amount of α2 in KHOS-240 cells was 60–80% of that in KHOS-NP or 40–50% of that in HOS-MNNG cells. Accordingly, in KHOS-240 cells α2 integrin mRNA signal could be detected only after a longer

TABLE I
Expression of α2 and α3 integrin subunits in human osteosarcoma cell lines

Integrin subunits were immunoprecipitated with specific antibodies from five parallel [^{35}S]methionine steady state labeled cell culture plates. After electrophoretic separation and fluorography, integrin subunits were quantified from fluorograms with an image analyzer system. The figures show optical density (arbitrary units) after reduction of the background. Mean ± S.D.

Integrin subunit	HOS	HOS-MNNG	KHOS-NP	KHOS-240
α2	0 ± 0	3.7 ± 1.5	2.5 ± 0.4	2.0 ± 1.2
α3	1.5 ± 0.5	2.3 ± 0.6	2.0 ± 1.8	1.9 ± 0.8

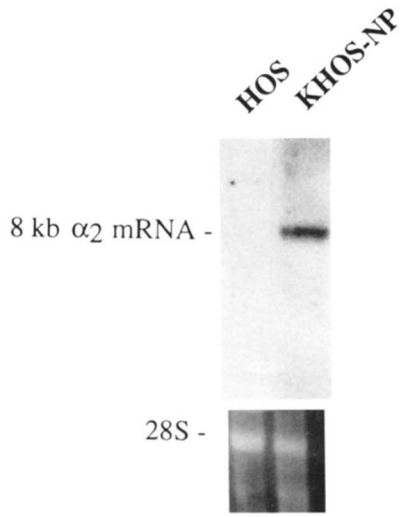

FIG. 3. **Northern blot analysis of α2 integrin subunit mRNA.** Total cellular RNA isolated from HOS or KHOS-NP cells were separated by gel electrophoresis, transferred to nylon membranes, and hybridized with a ^{32}P-labeled α2 integrin-specific cDNA probe. Ethidium bromide staining of the sample lanes shows equal loading.

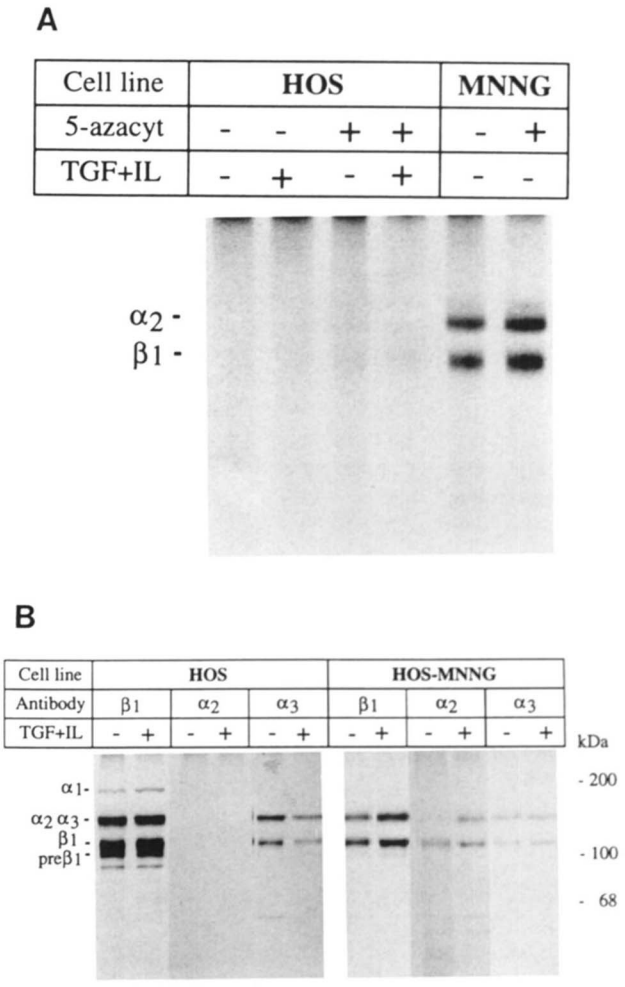

FIG. 4. **Effects of cytokines and a hypomethylating agent on the biosynthesis of α2 integrin subunit in HOS and HOS-MNNG cells.** *A*, HOS cells grown to 80% confluence were incubated in DMEM supplemented with 5% FCS in the presence of IL-1β (10 units/ml) and TGF-β1 (100 pM) with or without 5-azacytidine (10 μM) for 48 h, the last 24 h in methionine-free medium containing 50 μCi/ml [^{35}S]methionine. Cells were harvested, and aliquots of detergent-soluble cell extracts were immunoprecipitated with antibody against the α2 integrin subunit. Immunoprecipitated proteins were analyzed by gel electrophoresis and fluorography. *B*, HOS and HOS-MNNG cells were incubated with or without IL-1β (10 units/ml) and TGF-β1 (100 pM) for 22 h in serum-free medium, the last 8 h in the presence of 50 μCi/ml [^{35}S]methionine in methionine-free medium. Aliquots of detergent-soluble cell extracts were immunoprecipitated with antisera against α2, α3, or β1 integrin subunits. Immunoprecipitated proteins were analyzed by gel electrophoresis and fluorography.

exposure, and it was 10 or 30% of that in KHOS-NP or HOS-MNNG cells, respectively (Fig. 8). Changes in other integrin α subunits in KHOS-NP cells when compared to HOS cells were: α1, increased expression seen only in two out of five experiments; α5, about 2.5-fold increased; α6 about 3-fold increased. Changes in HOS-MNNG cells were: α1, decreased 50–70% (range in three measurements); α5, about 1.5-fold increase; α6, 3-fold increase. α1 expression was estimated from the amount of α1 integrin subunit co-precipitating with β1.

To further analyze the strong induction of α2 integrin expression after both chemical and viral transformation we measured the corresponding mRNA levels. In HOS cells, no α2 mRNA was found even after a long overexposure, whereas in KHOS-NP and HOS-MNNG cells a strong signal was seen (Figs. 3 and 8). Thus, we suggest that in HOS cells the expression of α2 is blocked at the transcriptional level. It has been shown, that numerous genes in permanent cell lines are stably inactivated due to *de novo* methylation of their promoters (Bird, 1992). We made several experiments to induce α2 integrin expression with 5-azacytidine. However, this hypomethylating agent was not able to turn on α2 integrin expression (Fig. 4*A*). Furthermore, HOS cells stayed negative for α2 integrin also after TGF-β1 and IL-1β treatment (Fig. 4, *A* and *B*). We have previously shown that these cytokines, especially when combined, are strong stimulators of α2 expression in MG-63 cells (Santala and Heino, 1991). Interestingly, in HOS-MNNG cells TGF-β/IL-1β combination increased α2 expression (Fig. 4*B*). Thus, we suggest that, although both the stimulation of α2 expression by cytokines and the induction α2 in transformation can be due to transcriptional regulation, their molecular mechanisms are not identical. α3 expression was slightly reduced in cytokine treated HOS cells (Fig. 4*B*), as we have previously shown in MG-63 cells (Heino and Massagué, 1989). However, in HOS-MNNG cells cytokine treatment had no effect on α3 expression (Fig. 4*B*).

Size of Intracellular Precursor β1 Integrin Pool Does Not Correlate with Tumorigeneity, but Regulates Maturation Rate of α3 Subunit—In addition to changes in the expression of specific α integrin subunits, another transformation-dependent alteration in integrin pattern was the reduction of intracellular preβ1 integrin pool in HOS-MNNG and KHOS-240 cells (Fig. 2*A*; Table II). After a steady-state labeling, preβ1 pool in KHOS-NP cells was, however, even larger than in HOS cells (Table II). We have previously described the relative reduction of preβ1 integrin pool in TGF-β treated fibroblasts, and shown that it is due to increased preα/preβ ratio in endoplasmic reticulum (Heino *et al.*, 1989).

The reduction of the size of the preβ1 pool accelerated its maturation (Fig. 5, *A* and *B*). In HOS and KHOS-NP cells, the half-maturation rate of preβ1 molecules was more than 5 h, whereas in HOS-MNNG cells and in KHOS-240 cells the time was less than 2 h. Interestingly, the maturation time of α subunits was concomitantly increased. In HOS and KHOS-NP cells the half-maturation rate of the α3 subunit pool was about 60 min, whereas in HOS-MNNG cells the time was noticeably longer, about 120 min (Fig. 6, *A* and *B*). Similar deceleration of α3 maturation was detected in KHOS-240 cells (not shown). Thus, the size of preβ1 pool regulates the maturation kinetics of α3, and probably also the other α subunits.

The electrophoretic mobility of integrins was remarkably slower in tumorigenic cell lines when compared to HOS and KHOS-240 cells (Fig. 2, *A* and *B*). This phenomenon, often seen in transformed cells, has previously been explained by altered integrin glycosylation (Akiyama *et al.*, 1990; Van de Water *et al.*, 1988; Symington *et al.*, 1989).

TABLE II

The ratio of precursor β1 integrin pool to total β1 integrin pool in human osteosarcoma cell lines

Integrin subunits were immunoprecipitated with anti-β1 antiserum from five parallel [^{35}S]methionine steady state labeled cell culture plates. After electrophoretic separation and fluorography, the bands representing precursor β1 and mature β1 integrin subunits were quantified from fluorograms with an image analyzer system. The percent of preβ1 from total β1 pool (preβ1 + matureβ1) was calculated. Mean ± S.D.

	HOS	HOS-MNNG	KHOS-NP	KHOS-240
Preβ1/totalβ1 × 100%	34 ± 6	20 ± 5	73 ± 7	11 ± 2

Tumorigenic Cell Lines Show Divalent Cation-dependent Increased Cell Adhesion to Type I Collagen—The attachment of cells to different substrata was studied in pre-coated microtiter plate wells. After 45 min the non-adherent cells were washed out, and the adherent cells were stained with crystal violet. Cell adhesion was tested in two different serum-free minimal essential media. The first one contained 1.8 mM Ca^{2+} and 0.8 mM Mg^{2+} and the second one was Ca^{2+}-free containing 0.8 mM Mg^{2+}. Integrin-ligand interaction is dependent on divalent cations, and, for example, α2β1 binding to type I collagen requires Mg^{2+} but is inhibited by Ca^{2+} (Grzesiak *et al.*, 1992). Here, the tumorigenic cell lines showed significantly ($p < 0.05$) increased cell adhesion to type I collagen, when tested in Ca^{2+}-free medium (Fig. 7). In Ca^{2+}-containing medium all the cell lines tested showed equal attachment to type I collagen (not shown). There were no differences in cell adhesion to laminin, whereas the attachment of tumorigenic cell lines to fibronectin was constantly increased (Fig. 7). Anti-serum against β1-integrin subunit (Heino *et al.*, 1989) blocked cell adhesion to type I collagen suggesting that the process is mediated by β1-integrins (not shown).

Type I Collagen Gene Expression Is Suppressed in Tumorigenic Cell Lines, but Not in KHOS-240 Cells—In tumorigenic cell lines the induction of α2 integrin expression and increased cell adhesion to type I collagen were associated to a large decrease in collagen gene expression (Fig. 8). Collagen α1(I) mRNA levels were reduced about 90% and 85% in KHOS-NP and HOS-MNNG cells, respectively. Also cellular fibronectin mRNA levels decreased about 60% and 85%, respectively (Fig. 8). Interestingly, virally transformed but non-tumorigenic subclone KHOS-240 expressed similar α1(I) collagen and fibronec-

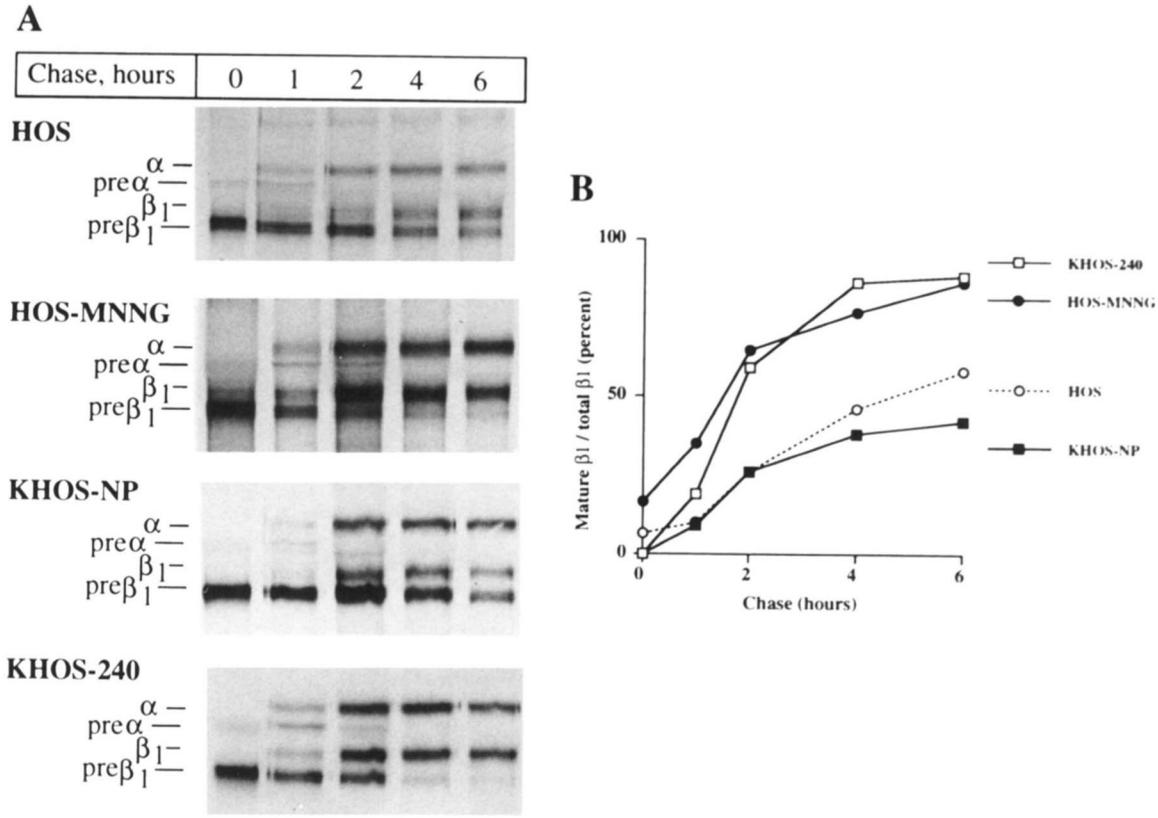

FIG. 5. **Maturation of β1 integrin subunit in human osteosarcoma cell lines.** Confluent cultures of HOS, HOS-MNNG, KHOS-240, and KHOS-NP cells were incubated in methionine-free medium for 3 h, the last 60 min in the presence of 100 μCi/ml [^{35}S]methionine. Cultures were then shifted to unlabeled serum-free medium for the indicated lengths of time (0, 1, 2, 4, and 6 h). Cells were harvested and aliquots of detergent-soluble cell extracts were immunoprecipitated with antiserum against β1 integrin subunit. Immunoprecipitated proteins were analyzed by gel electrophoresis and fluorography (*A*). Fluorograms were quantified by an image analyzer system (*B*).

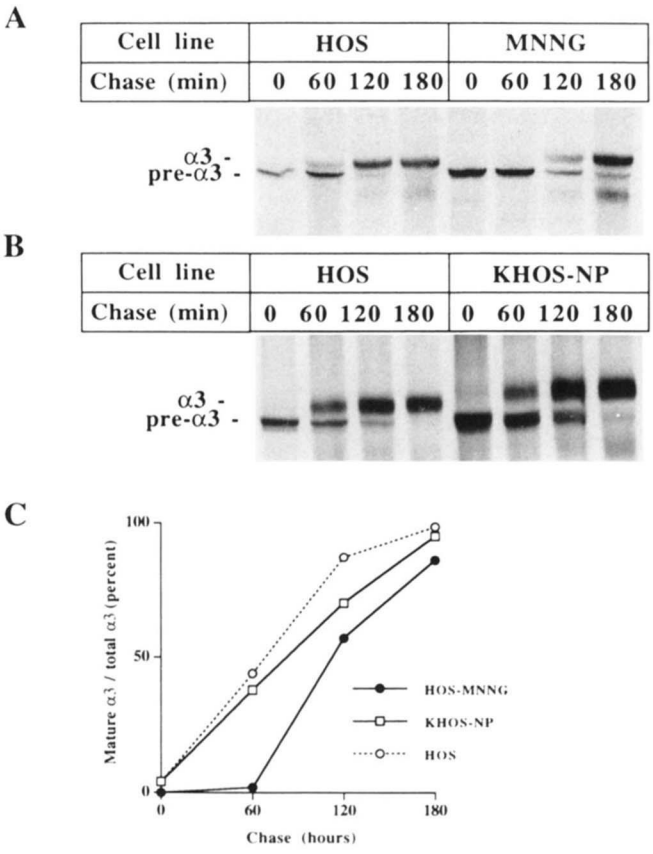

FIG. 6. **Maturation of α3 integrin subunit in HOS, KHOS-NP, and HOS-MNNG cells.** Confluent cultures were incubated in methionine-free medium for 4 h, the last 60 min in the presence of 100 μCi/ml [^{35}S]methionine. Cultures were then shifted to unlabeled serum-free medium for the indicated lengths of time. Cells were harvested and aliquots of detergent-soluble cell extracts were immunoprecipitated with antiserum against α3 integrin subunit. Immunoprecipitated proteins were analyzed by gel electrophoresis and fluorography (*A* and *B*). Fluorograms were quantified by an image analyzer system (*C*). In *C*, HOS represents mean values from experiments shown in *A* and *B*.

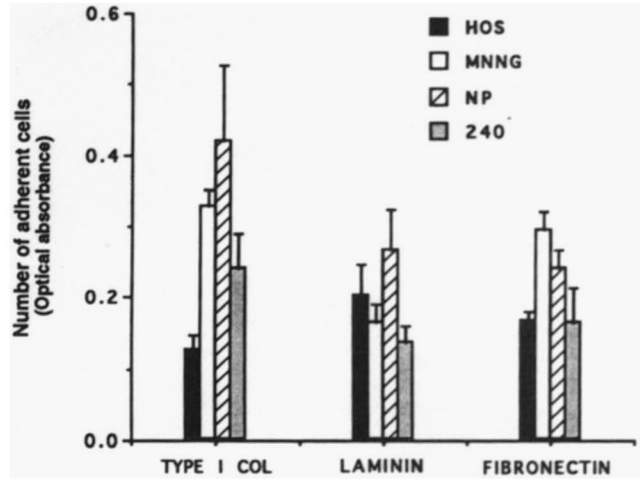

FIG. 7. **Attachment of osteosarcoma cells to different substrata.** Microtiter plate wells were precoated with type I collagen (*COL*), laminin (*LM*), or fibronectin (*FN*). Cells were allowed to attach for 45 min in Ca^{2+}-free medium containing Mg^{2+} (0.8 mM). Adherent cells were stained and their number was estimated by measuring optical absorbance. The data are mean ± S.D. from four parallel experiments.

tin mRNA levels to those of HOS cells. mRNA levels of some other matrix proteins, for example a proteoglycan, biglycan, were constant in all cell lines (not shown).

DISCUSSION

Cancer cell migration and invasion, as well as the formation of metastasis, are phenomena where cell-matrix interactions are of fundamental importance. Thus, transformation-related changes in the expression or function of the cell adhesion proteins can be essential for the tumor behavior (Ruoslahti, 1992). Our approach was to study the regulation of matrix protein and integrin gene expression in HOS cells and in their virally transformed counterparts. These cells were also compared to HOS cells chemically transformed with MNNG. We wanted to determine whether there are changes common to all transformed HOS cell lines. We screened the synthesis of α1, α2, α3, α5, and α6 subunits and found that α2β1 is not produced in HOS cells, whereas it was strongly expressed in HOS-MNNG and KHOS-NP cells and in addition to that in KHOS-240 cells a weaker expression was detected. α2β1 is a collagen receptor, which can in many cell lines bind also laminin (Languino *et al.*, 1989). Previous studies have shown that α2β1 is essential for the type I collagen reorganization and collagen gel contraction by fibroblasts (Schiro *et al.*, 1991; Klein *et al.*, 1991a). Also here, the tumorigenic cell lines, in which the α2 expression was induced, showed increased cell adhesion to type I collagen. This was seen in cell adhesion assays performed in the presence of Mg^{2+} but not in the presence of Ca^{2+}. Grzesiak *et al.* (1992) have reported that Mg^{2+} supports the α2β1-collagen interaction, whereas Ca^{2+} inhibits it. Type I collagen is the major component of bone matrix, and it is tempting to speculate that the cell-collagen interaction may be of importance in the formation of osteogenic cancer.

The importance of cell-collagen interaction was also suggested by our finding that type I collagen mRNA levels were strongly down-regulated in tumorigenic cell lines. The decreased matrix protein synthesis has been connected to transformed cell phenotype also by other investigators (Slack *et al.*, 1992). Here, it was, however, associated with the increased cell adhesion to collagen and with the appearance of a new collagen receptor. We suggest that the reduced ability to produce its own matrix and a new mechanism to interact with bone matrix might be essential features for tumorigenic derivatives of HOS cells. This was supported by the fact that these changes were seen in tumorigenic cells, not depending on the mechnism used to transform the cells. Furthermore, in virally transformed but non-tumorigenic KHOS-240 cells collagen mRNA levels were not changed, and the induction of α2 integrin was smaller.

Previously, in other cancer types α2β1 integrin has been associated to the transformed phenotype. A relative overexpression of α2 subunit has been reported in melanomas (Klein *et al.*, 1991b) and in non-small cell lung cancer (Chen *et al.*, 1991). Furthermore, in rhabdomyosarcoma cells α2 expression is required for the formation of experimental metastasis *in vivo* (Chan *et al.*, 1991). Several other transformation-related changes in integrin expression have been reported. Plantefaber and Hynes (1989) showed that in the viral transformation of rodent cells most β1-associated integrin α chains are down-regulated. Only α3 expression stayed constant. These observations are in accordance with much older findings that fibronectin often disappears from cell surface in transformation (Ruoslahti, 1992). However, in many similar transformation models using different cell types no disappearance of fibronectin receptors has been seen (Akiyama *et al.*, 1990; Ylänne and Virtanen, 1989). The melanoma cells have been reported to express α7 and αv integrins unlike their non-transformed counterparts (Kramer *et al.*, 1991; Felding-Habermann *et al.*, 1992). Thus, the conclusion is that the transformation-related changes in the cellular integrin pattern are very much depend-

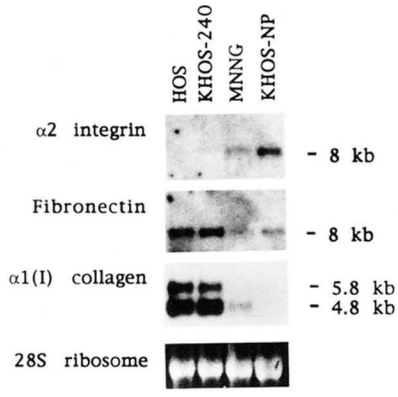

FIG. 8. **Cellular mRNA levels of α1(I) collagen and fibronectin.** Total cellular RNAs isolated from different cell lines were separated by gel electrophoresis, transferred to nylon membranes, and hybridized with ^{32}P-labeled cDNA probes specific to α2 integrin, fibronectin, and collagen α1(I). Ethidium bromide staining of the larger ribosomal subunits shows the equal loading of gels.

ent on the cell type and no general rules can be found.

The fact that α2 integrin is not expressed in HOS cells and that both chemical and viral transformation can turn on its expression makes these osteosarcoma cell lines a good model to study the regulation of integrin gene expression. In addition to cancer, integrin expression is regulated in other physiological and pathological conditions, including cell differentiation (Adams and Watt, 1990), chronic inflammation (Nikkari *et al.*, 1993), and wound healing (Larjava *et al.*, 1993). TGF-β1 can induce the synthesis of most integrin subunits, but its effects are cell type-specific (Ignotz and Massagué, 1987; Heino *et al.*, 1989; Ignotz *et al.*, 1989). We have shown that in certain cell lines, for example in MG-63 cells, TGF-β1 stimulates some and concomitantly down-regulates other α subunits (Heino and Massagué, 1989). Other growth factors and cytokines known to regulate integrin synthesis include IL-1β, tumor necrosis factor-α, interferon-γ, and nerve growth factor (Rossino, 1990; Santala and Heino, 1991; Defilippi *et al.*, 1991). TGF-β seems to up-regulate and down-regulate integrin synthesis at the mRNA level (Heino and Massagué 1989), whereas Defilippi *et al.* (1991) could not find changes in the corresponding mRNA levels when they used a interferon-γ/tumor necrosis factor-α combination to decrease β3 protein synthesis in endothelial cells. Thus, integrin expression might be regulated at two different levels. Here, we suggest transcriptional regulation because no α2 mRNA was found in HOS cells. Furthermore, 5-azacytidine treatment did not turn on this gene; it is supposed that it is not suppressed because of cell culture-generated *de novo* promoter methylation (Bird 1992). The regulatory elements in integrin genes are still incompletely known. Nucleotide sequences of the 5′-flanking regions in at least five integrin subunits, namely β1, αM, αIIb, α4, and α5, have been published (Birkenmeier *et al.*, 1991; Rosen *et al.*, 1991, Cervella *et al.*, 1993; Shelley and Arnaout, 1991; Uzan *et al.*, 1991). They show the presence of multiple putative regulation sites. However, much more data are required before the specific transcription factors involved in the process can be named. Our data suggest the presence of a strong suppressive element in HOS cells. Interestingly this element seems to be transformation-sensitive. The data also support the idea that the integrin subunits can be regulated independently. Furthermore, in spite of the fact that both cytokines and transformation stimulate α2 integrin expression, the mechanism of their action is not identical.

In many cell lines β1 integrin is produced in large excess, when compared to α subunits (Ignotz and Massagué, 1987; Akiyama and Yamada, 1987; Heino *et al.*, 1989). Most of the β1 molecules stay as immature precursors in endoplasmic reticulum. Furthermore, heterodimer formation is required before integrins are transferred further in the maturation pathway and finally to the cell surface (Heino *et al.*, 1989). The preβ1 molecules, which do not form a complex with preα molecules, will be directed to the intracellular degradation pathway. Our studies with TGF-β1 have also shown that the number of integrins on the cell surface is regulated by the α chain production (Heino *et al.*, 1989). Here, we could show the reduction of preβ1 pool after both viral (KHOS-240) and chemical (HOS-MNNG) transformation. However, in KHOS-NP cells the size of the preβ1 pool was even larger than in HOS cells. This suggests that the phenomenon is not required for tumorigenic phenotype of HOS cells. The reduction of intracellular preβ1 has been described also in other transformation models (Akiyama *et al.*, 1990). Here, we show that it leads to situation where β1 molecules spend noticeably shorter and α chains longer time as precursors after translation. An interesting question is whether the function of integrins is in any way dependent on the kinetics of the maturation process. This question cannot yet be answered. However, it is known that the integrin function is very sensitive to conformational changes (Masumoto and Hemler, 1993). In tumorigenic osteosarcoma cells mature integrin subunits had a noticeably altered electrophoretic mobility. This phenomenon has been connected also previously to transformed cell phenotype and it has been proposed to be due to altered integrin glycosylation (Symington *et al.*, 1989; Van de Water, 1988; Akiyama *et al.*, 1990), again supporting the idea that integrin maturation can be disturbed in transformation. Interestingly, the non-tumorigenic KHOS-240 cell line did not differ from HOS cells in this regard.

To conclude, we have described that both viral and chemical transformation have very similar effects on HOS cell phenotype. After transformation the tumorigenic cell lines have reduced their type I collagen mRNA levels dramatically and altered the mechanism they use to interact with type I collagen. These results suggest a linkage between collagen and collagen receptor gene expression and propose also the importance of cell-collagen interaction in the formation of osteogenic malignancies.

Acknowledgments—We thank Drs. Martin Hemler, Eero Vuorio, and Sen-itiroh Hakomori for cDNAs; Drs. Virgil Wood, Caroline Damsky, Kenneth Yamada, and Arnould Sonnenberg for antibodies; and Dr. Joan Massagué for TGF-β1. We are grateful to Jorma Hermonen, for preparation and purification of synthetic peptides, and Hans Helenius, for statistical analyses. The expert technical assistance of Liisa Peltonen, Leila Saarinen, and Marita Potila is gratefully acknowledged.

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