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α4 Integrin Increases Anoikis of Human Osteosarcoma Cells

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Abstract Cell motility, growth, and proliferation are regulated by adhesion to the extracellular matrix. Detachment of adherent cells from extracellular matrix results in induction of apoptosis (''anoikis''). Transformed cells often show an anchorage-independent growth that enables them to acquire a motile, invasive phenotype. This phenotype has been associated with the altered expression and function of the integrin family of transmembrane proteins that mediate cell adhesion to the extracellular matrix. Although α4 integrin is normally expressed on leukocyte subpopulations, a number of metastatic melanomas and sarcomas express it as well. In this study, we demonstrated the expression of α4 integrins on the human osteosarcoma cell line SAOS and on metastatic osteosarcoma lesions from the lung and pericardium. We further demonstrated that α4 integrin is coupled to the β1 subunit by biochemical analysis and by using a mAb directed against a combinatorial epitope unique to the α4β1 molecule. SAOS cells undergo anoikis when adherence is denied. Anoikis involved the activation of caspase 3 and the release of cytochrome c from mitochondria. Treatment of non-adherent SAOS with an anti-α4 mAb increased anoikis while anti-β1 integrin mAbs did not alter anoikis, thus indicating a novel function for the α4 subunit in the control of cell death. Since integrins can control cell migration, proliferation, and apoptosis these results demonstrate a potential role for α4 integrin during multiple aspects of osteosarcoma metastasis. J. Cell. Biochem. 88: 1038–1047, 2003.

Key words: apoptosis; extracellular matrix; osteogenic sarcoma; cell adhesion; metastasis

Cell adhesion has a complex relationship with cancer and, ultimately, the diagnosis and management of this disease will require an understanding of this association. After normal cells become transformed many events connected with adhesion can occur such as loss of contact inhibition of growth, detachment from other cells and extracellular matrix, invasion of surrounding tissue, extravasation, and metastasis to distinct anatomical sites. Adhesion not only regulates the location and motility of tumor cells, but growth and proliferation can also be controlled by adhesive mechanisms. An obvious mechanism for growth regulation would be the tissue specific homing of metastatic cells to a region containing appropriate growth factors. Undeniably this can occur, but it has recently become clear that cell adhesion receptors can transmit intracellular signals that can positively or negatively regulate cell growth and arrest [Schwartz and Assoian, 2001]. Another mechanism for adhesion control of growth and metastasis is anoikis. Anoikis is defined as cell death induced by the lack of adhesion. It was first described in endothelial and epithelial cells where it maintains a dynamic balance of cell turnover [Meredith et al., 1993; Frisch and

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Francis, 1994]. Resistance to anoikis has been associated with malignancy, since it affords the cells increased survival time in the absence of matrix, facilitating their migration and eventual colonization of secondary sites [Yawata et al., 1998; Streuli and Gilmore, 1999; Shanmugathasan and Jothy, 2000]. Thus the identification and characterization of tumor cell adhesion receptors that control cell cycle arrest, apoptosis, or proliferation may lead to the development of anti-cancer strategies.

Integrins are a family of heterodimeric transmembrane receptors that bind to various extracellular and cell surface proteins during adhesion, migration, and homing of normal and neoplastic cells [Meredith et al., 1996]. Furthermore, integrins may play a role in the ability of tumor cells that have become nonadherent to survive and metastasize [Frisch and Screaton, 2001]. Due to this central role in normal and pathologic cellular physiology, the determination of integrin expression patterns in normal and diseased cells should be useful as diagnostic and prognostic markers and provide attractive targets for developing therapeutic drugs.

Osteosarcoma is the most common malignant primary bone tumor. Up to 80% of patients show macroscopic and microscopic pulmonary metastases at presentation. Effective adjuvant chemotherapy has improved long-term disease free 5-year survival rates from 10 to 20% in 1970s to nearly 60–70% [Link et al., 1986; Sluga et al., 1999]. Treatment of patients with relapsed disease requires intensive chemotherapy with limited efficacy [Goorin et al., 1991; Ferguson and Goorin, 2001]. New therapeutic approaches are therefore needed to improve patient survival. Studying the cellular mechanisms involved in the metastatic process may provide alternative treatments that may prevent or reduce tumor cell migration.

An experimental in vitro model using the human osteosarcoma cell line, SAOS-2 (SAOS) was used in our studies. SAOS cells were derived from a primary osteosarcoma lesion from an 11 year-old girl [Fogh et al., 1977]. SAOS cells are unable to form metastases in nude mice, however, multiple in vivo passages result in variants with high metastatic potential [Jia et al., 1999].

Expression of α4 integrins in the human adult is normally restricted to monocytes, and B and T lymphocytes but during the tumorigenic process a number of cell types such as metastatic melanomas and sarcomas express the α4 integrin [Schweighoffer et al., 1993; Erle et al., 1994; Holzmann et al., 1998]. In this study, we showed the expression of α4 and β1 integrin subunits in human osteosarcoma metastatic lesions and in SAOS cells. Furthermore, because modulation of integrin function has different effects on cell fate, the role of α4 integrin during anoikis of SAOS cells was also investigated. We found that the addition of soluble mAbs against the α4 integrin enhanced anoikis in suspended SAOS cultures, thus suggesting a unique role for α4 integrins in controlling cell death.

MATERIALS AND METHODS

Cell Culture and Tissue

The human osteosarcoma cell line SAOS-2 (SAOS) was obtained from the American Type Culture Collection (Manassas, VA). SAOS cells were maintained in Eagle's MEM (BioWhittaker, Walkersville, MD), 2 mM L-glutamine, 1 mM sodium pyruvate and non-essential amino acids (Sigma, St. Louis, MO). SAOS cells were cultured for 24 h at 37°C with or without soluble mAbs (10 μg/ml) on untreated tissue culture plastic or on tissue culture plastic coated with poly (2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma) at a density of 5 mg/cm^2. Poly-HEMA was prepared by dissolving it in 95% ethanol to a concentration of 50 mg/ml. After poly-HEMA was added, wells were allowed to dry overnight under sterile conditions in a laminar flow hood. Tissue samples were obtained from a 22-year-old patient diagnosed with fibroblastic osteosarcoma of the right distal femur. Tissue samples from metastatic lesions of the lung (0.831 g) and the pericardium (1.13 g) were obtained after left thoracotomy performed 2 years after diagnosis.

Monoclonal Antibodies

Monoclonal antibodies used in these studies were: anti-α1 mAb TS2/7 [Hemler et al., 1984], anti-α2 mAb P1E6, anti-α3 mAb P1B5 [Wayner et al., 1988], anti-α4 mAbs 90B8, L25, B5G10 [Hemler et al., 1984, 1987; McIntyre et al., 1989], anti-α5 mAb P1D6 [Wayner et al., 1988], anti-α6 G0H3 [Sonnenberg et al., 1987], anti-β1 mAbs 18D3, 33B6, and 87B9 [Bednarczyk et al., 1992, 1993], anti-β4 mAb 439-9B [Falcioni et al., 1988], anti-α4β1 mAb

19H8 [Bednarczyk et al., 1994], anti-α4β7 mAb ACT-1 [Schweighoffer et al., 1993], anti-CD26 mAb AC7 [Bednarczyk et al., 1991], anti-human MHC Class I mAb W6/32 [Barnstable et al., 1978] and anti-T cell receptor mAb T40/25 [Kappler et al., 1983]. All mAb were mouse anti-human except the rat anti-human GoH3.

Iodination of Purified mAb

Purified mAb were radioiodinated using 1,3,4,6-tetrachloro-3α,6α, diphenylglycoluril (IODO-GEN, Pierce Chemical Co). Briefly, 25 μg of purified mAb in 100 μl of phosphate buffered saline (PBS) was added to an IODO-GEN coated 12 × 75 mm glass tube (10 μg of IODO-GEN/tube). The iodination reaction was initiated by the addition of 500 μCi of $Na^{125}I$ (Du Pont-New England Nuclear) and allowed to continue for 15 min at room temperature. The reaction was stopped by removal of the mAb sample from the tube. ^{125}I-mAb was separated from free iodine by gel filtration on Sephadex G-25 fine matrix (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) equilibrated in PBS with 0.5% bovine serum albumin (Intergen Purchase, NY) and 0.02% sodium azide.

Iodination of Cell Surface Proteins and Immunoprecipitation

Cell surface proteins were labeled by lactoperoxidase-catalyzed radioiodination by a modification of a procedure described previously [Keski-Oja et al., 1977]. Briefly, $2–3 \times 10^6$ cells were washed three times in PBS and once in PBS with 1 μM potassium iodide (KI). Cells were resuspended in 500 μl of PBS with 1 μM KI and 10 μg of lactoperoxidase (Sigma) and 0.2 IU of glucose oxidase (Sigma) were added. Next, 1.0 mCi of $Na^{125}I$ in 500 μl of PBS with 1 μM KI and 10 mM glucose was added and the sample was incubated at room temperature. After 15 min the cells were washed three times in ice-cold PBS containing 5 mM KI. ^{125}I-labled cells were lysed in lysis buffer (150 mM NaCl, 10 mM Tris-HCI, pH 8.0 containing protease inhibitors: leupeptin at 10 μg/ml, pepstatin A at 1 μg/ml, aprotinin at 10 μg/ml, and sodium azide 0.02% v/v (Sigma) with 2% CHAPS (Sigma)) for 1 h on ice. Clarified lysate was precleared with fixed *S. aureus* (Sigma) and then immunoprecipitated with protein A agarose beads (Roche, Indianapolis, IN) precomplexed with goat anti-mouse immunoglobulins (ICN, Costa Mesa, CA) and relevant mAb, by incubation at 4°C for 16 h. Polypeptides were eluted by boiling in reducing Laemmli sample buffer, and separated by 7.5% SDS–PAGE. Dried gels were exposed to Kodak XR film at −80°C.

Binding Assay

Purified mAb (20 μg/ml PBS) was added to the wells of a polyvinyl chloride microtiter plate (50 μl/well) and incubated for 12 h at 4°C. Excess antibody was aspirated, and the plates were washed three times with PBS. Each well was treated with 250 μl of 0.5% BSA/PBS for 4 h at room temperature, and the unbound BSA was removed. Tissue samples from a left upper lobe lung osteosarcoma (0.831 g) and from the pericardium (1.13 g) were lysed in 1% Triton, 150 mM NaCl, 10 mM Tris-HCI, 0.02% v/v sodium azide pH 7.5 with or without 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$ for 1 h on ice. BSA/PBS was added to lysates to a final concentration of 5 mg/ml, and 50 μl of this mixture was added to each well. After incubation for 5 h at 4°C, the wells were washed four times with the appropriate lysis buffer diluted with an equal volume of 0.1% BSA/PBS (wash buffer). Radioiodinated antibody (1×10^6 cpm/50 μl of wash buffer) was added to each well and incubated for 1 h at 4°C. The plates were washed five times with a buffer to remove unbound radioactivity, and the wells were counted for bound radioactivity in a LKB 1272 CliniGamma gamma counter (Pharmacia, Turku, Finland). Each data point represents the average from triplicate samples.

Cell Surface Staining and Flow Cytometry

Cells were washed twice with fluorescence activated cell sorter (FACS) buffer (PBS containing 0.1% FCS, 0.02% sodium azide), and incubated for 1 h at 37°C with 1 μg/ml purified mAb. Following two washes in FACS buffer, cells were incubated with 1:100 dilution of FITC-conjugated goat anti-mouse IgG (ICN) or PE-conjugated goat anti-rat IgG (Caltag Laboratories, Burlingame, CA) for 1 h at 4°C. Following two washes with FACS buffer, fluorescence was analyzed on an Epics Profile flow cytometer (Coulter, Miami, FL).

Cell Cycle Analysis

Cell cycle analyses were performed using propidium iodide (PI) staining with subsequent

FACS analysis. Cells/well (5×10^5) were cultured either on plastic or poly-HEMA treated 6 well tissue culture plates for 24 h at 37°C in a 5% CO_2 atmosphere. After incubation, adherent cells were detached with trypsin (0.5% trypsin/0.1% EDTA in PBS). Cells were harvested in complete EMEM medium and centrifuged at 500*g* for 10 min. Pellets were washed with PBS and fixed with cold 75% ethanol overnight at 4°C. After fixation, cells were washed with PBS and stained with 500 μl of PI solution (50 μg/ml in PBS). Cells were incubated at 37°C for 30 min and analyzed by flow cytometry on an Epics Profile flow cytometer (Coulter).

Cytochrome c Release Measurements

Cytochrome c release from mitochondria was determined by Western immunoblot. Treated and untreated SAOS cells were harvested and centrifuged at 500*g* for 5 min. Pellets were gently lysed for 5 min in ice-cold lysis buffer (25 mM Tris and 5 mM $MgCl_2$, pH 7.4). Lysates were centrifuged for 5 min at 16,000*g*. Supernatants from equal number of cells (1×10^6) were mixed with 1 × Laemmli reducing buffer and resolved by 15% SDS–PAGE. Polypeptides were transferred to nitrocellulose membranes (0.2 μM, Schleicher & Scheull, Keene, NH). Cytochrome c was detected by immunoblotting with the monoclonal antibody clone 7H8.2C12 (Pharmingen, San Diego, CA).

Capase-3 Activity Assay

Treated and untreated SAOS cells (1×10^6) were lysed in 100 μl lysis buffer (100 mM HEPES, 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 10 mM DTT, 2 mM PMSF, 100 μM pepstatin, 10 μg/ml leupeptin, pH 7.5). Lysates were incubated at 4°C overnight. After incubation, 900 μl of reaction buffer (100 mM HEPES, 10% sucrose, 0.1% CHAPS, 1 mM EDTA) was added to each sample. Two microliters of 25 mM solution (in DMSO) of fluorigenic substrate specific for caspase 3 (Ac-Asp-Glu-Val-Asp-AMC ammonium salt) were added. Samples were incubated at 37°C for 1 h and analyzed at excitation 380 nm and emission 480 nm (RF-1501 spectrofluorimeter Shimadzu Scientific Instruments, Columbia, MD). Relative fluorescence was calculated by subtracting the blank fluorescence (buffer plus substrate) from the experimental fluorescence.

RESULTS

Human Osteosarcoma Metastatic Lesions of the Lung and Pericardium Express α4 Integrin Subunit in Association With the β1 Subunit

A solid phase double determinant binding assay was used to detect the expression of α4 integrin in Triton X-100 lysates of osteosarcoma metastatic lesions of the lung and pericardium. Radioiodinated anti-α4 mAb L25 was used to detect antigens bound by capture antibodies immobilized in plastic wells. No mAb L25 was captured by antigens presented by BSA, the control anti-T cell receptor mAb T40/25, or anti-CD26 mAb AC7 (Fig. 1). In contrast, the mAb B5G10, which binds to an α4 epitope that is spatially distinct from the mAb L25 binding site, clearly binds and presents the integrin α4 to mAb L25.

The results in Figure 1 demonstrate that human osteosarcoma cells express the integrin α4 subunit. In order to confirm that this alpha subunit is associated with the β1 subunit, cells were lysed with Triton X-100 containing divalent cations to preserve integrin subunit integrity, and binding assays performed with radioiodinated anti-α4 mAb L25. Again, no mAb L25 was captured by antigens presented by BSA, the control anti-T cell receptor mAb T40/25, or anti-CD26 mAb AC7 (Fig. 2). When the

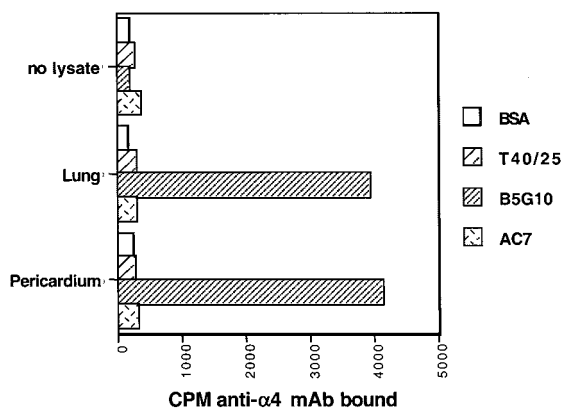

Fig. 1. Expression of integrin α4 on human osteosarcoma. mAb or BSA adsorbed to wells of a microtiter plate were used to capture antigens from 1% Triton lysates of metastatic lesions of pericardium and lung. Radioiodinated anti-α4 mAb L25 was used to detect bound antigens as described under Materials and Methods. The capture antibodies used were the anti-T cell receptor mAb T40/25, the anti-α4 subunit specific mAb B5G10, and the anti-CD26 mAb AC7. Data are expressed as the average ± SEM from triplicate determinations. Where error bars are not indicated, SEM was smaller then the data point symbol.

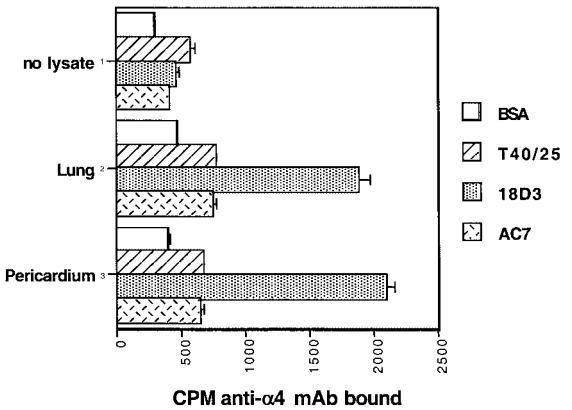

Fig. 2. Integrin α4 subunit is associated with the integrin β1 subunit on human osteosarcoma. mAb or BSA adsorbed to wells of a microtiter plate were used to capture antigens from 1% Triton (plus divalent cations) lysates of metastatic lesions of pericardium and lung. Radioiodinated anti-α4 mAb L25 was used to detect bound antigens as described under Materials and Methods. The capture antibodies used were the anti-T cell receptor mAb T40/25, the anti-β1 subunit specific mAb 18D3, and the anti-CD26 mAb AC7. Data are expressed as the average ± SEM from triplicate determinations. Where error bars are not indicated, SEM was smaller then the data point symbol.

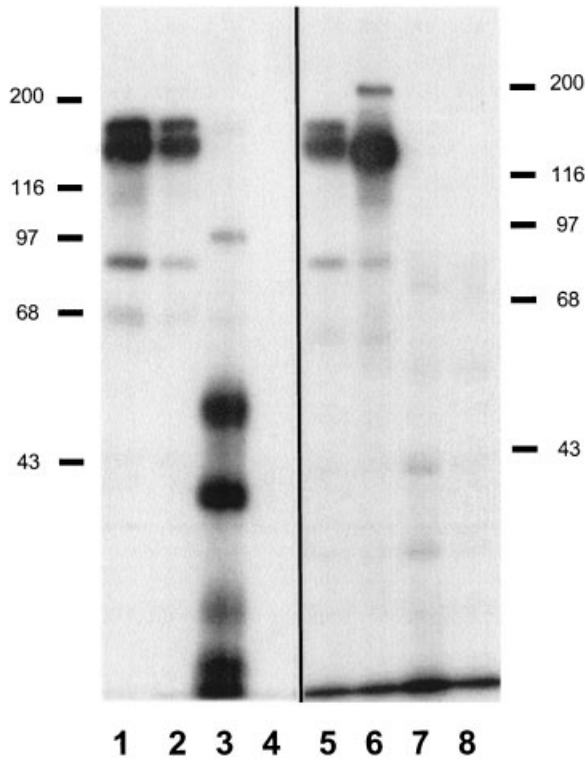

Fig. 3. Immunoprecipitation of α4 and β1 integrins from the human osteosarcoma cell line SAOS. Radioiodinated HPB-ALL (**lanes 1–4**) and SAOS (**lanes 5–8**) cells were solubilized with detergent and polypeptides were immunoprecipitated with anti-α4 mAb L25 (lanes 1 and 5), anti-β1 mAb 18D3 (lanes 2 and 6), anti-T cell receptor mAb T40/25 (lanes 3 and 7), and anti-α2 mAb P1E6 (lanes 4 and 8).

anti-β1 integrin subunit mAb 18D3 was used to capture antigens, radiolabeled L25 could now be detected. These results show that metastatic human osteosarcoma cells express the integrin α4 in association with the β1 subunit. Expression of the α4β1 integrins was also found in primary osteosarcoma tumors by flow cytometry (data not shown).

Expression of the Integrin α4β1 by the SAOS Human Osteosarcoma Cell Line

Human SAOS osteosarcoma cells were examined for cell surface expression of the integrin α4β1 by immunoprecipitation from detergent lysates of radioiodinated cells (Fig. 3). When immunoprecipitated with an anti-α4 mAb, the control $\alpha4\beta1^+$ HPB-ALL human T leukemic cells showed the α4 subunit at 150 kDa, the α4 fragments of 85 and 70 kDa, and the β1 subunit at 130 kDa (lane 1). An identical pattern was obtained with the anti-β1 mAb (lane 2) indicating that the integrin α4β1 is the major β1 integrin family member on these cells. With SAOS cells, α4 in association with β1 was clearly seen (lane 5). The β1 specific mAb precipitates α4β1 and another higher molecular weight alpha subunit of approximately 200 kDa, probably the integrin α1 subunit (lane 6). The integrin α2 specific mAb did not immunoprecipitate a significant amount of radiolabeled proteins from both HPB-ALL and SAOS cells (lanes 4 and 8).

SAOS cells were examined for the expression of a variety of integrin subunits by flow cytometry to confirm the results of Figure 3. As shown in Table I, the integrin subunits α1, α4, and β1 appear to be highly expressed integrins on these cells when considering both percent

TABLE I. FACS Analysis of SAOS Integrins

	% +	M.F.I.
FITC control	2.1	0.49
PE control	1.9	0.63
α1	51.4	3.85
α2	5.1	0.88
α3	23.2	1.86
α4	72.8	6.24
α5	18.7	1.73
α6	16.4	1.48
β1	74.9	11.20
β4	3.3	0.61
α4β1	63.0	5.22
α4β7	2.5	0.52

positive and mean fluorescence intensity (MFI). These results also indicate that SAOS cell express the integrin α4β1 instead of α4β7. Altogether the results from the Figure 3 and Table I indicate that the two major β1 integrins on SAOS cells are α1β1 and α4β1.

SAOS Undergo Apoptosis When Adherence is Denied

A significant fraction of SAOS cells underwent apoptosis (anoikis) after adherence was denied by culture in poly-HEMA treated plates (Fig. 4). Apoptosis was determined by PI staining followed by flow cytometry analysis. Figure 4 shows 51.8% of SAOS cells in the sub-G0/G1 apoptotic phase after culture in non-adherent conditions, compared to 10.3% in the adherent conditions.

Anoikis of SAOS Involves the Release of Cytochrome c From Mitochondria and Activation of the Caspase Cascade

To further investigate the mechanism by which anoikis occurs, the involvement of cytochrome c release from mitochondria and the subsequent activation of the caspase cascade was studied. Increased levels of cytochrome c, as shown in Figure 5 (panel A), were detected by Western immunoblot in the cytoplasm of SAOS cells after culture in suspension. Caspase 3 activity was determined using a specific fluorogenic substrate, which upon protease cleavage releases a fluorogenic product that can be quantitated by a spectrofluorimeter. Caspase 3 activity of SAOS cells cultured in suspension was significantly higher ($P \leq 0.0017$) compared to cells allowed to attach to the culture well (Fig. 5, panel B). These results indicate that anoikis of SAOS involves the release of cytochrome c from mitochondria and the activation of the caspase cascade.

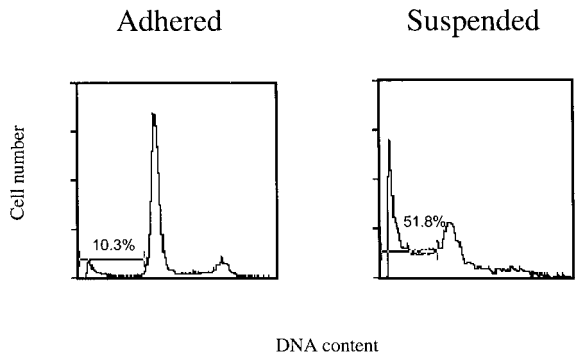

Fig. 4. SAOS cells undergo apoptosis when adherence is denied. SAOS cells were allowed to adhere to plastic or remain suspended in poly-HEMA coated cell culture wells for 24 h. After culture, cells were fixed and stained with PI. DNA content was analyzed by flow cytometry.

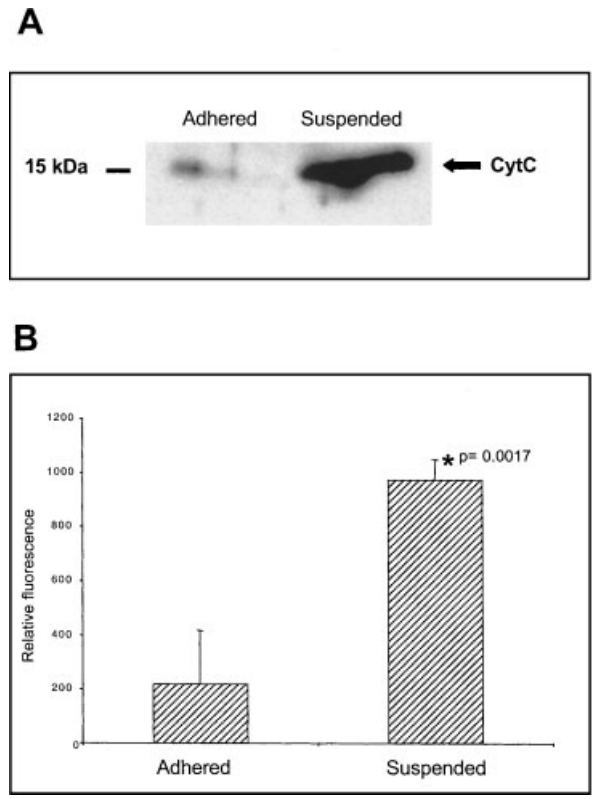

Fig. 5. Death of SAOS in the absence of adhesion involves cytochrome c release and activation of caspase 3. **A**: Cytochrome c release from mitochondria was detected by immunoblotting 24 h after culture on plastic (**lane 1**) or on poly-HEMA coated cell culture wells (**lane 2**). **B**: Caspase 3 activity was determined using a specific fluorogenic substrate after a 24-h incubation on plastic (**lane 1**) or on poly-HEMA coated wells (**lane 2**). Results represent the average relative fluorescence (blank fluorescence minus sample fluorescence) of three independent experiments ± SD ($P = 0.0017$).

Anti-α4 mAb 90B8 Increases Anoikis of Suspended SAOS Cells

The relationship of the integrin α4β1 to SAOS anoikis was determined by incubating specific mAbs with cells for 24 h in adherent and suspended conditions. When SAOS cells are suspended with a mAb specific to the integrin α4 subunit (90B8), a 52% increase in anoikis was found. Other mAbs such as the non-binding IgG control mAb T40/25 or mAbs specific for MHC Class I (W6/32) and β1 integrin (33B6 and 87B9) did not increase the level of apoptosis (Fig. 6). In a dose response experiment with the anti-α4

mAb 90B8, increased apoptosis was demonstrated as low as 1.0 picogram (data not shown). The induction of apoptosis after treatment with mAb 90B8 can be observed as early as 6 h with maximum enhancement between 18 and 24 h after treatment (data not shown). Finally, this mAb did not induce apoptosis in adherent SAOS cells indicating that it was not a general cytotoxin (data not shown).

Since anoikis of SAOS involved the release of cytochrome c from mitochondria and the activation of the caspase cascade, we wanted to determine if the increased anoikis observed after treatment with mAb 90B8 correlated with an increase in cytochrome c release and caspase 3 activity. Figure 7 shows a modest increase in the levels of cytochrome c (panel A) and caspase 3 activity (panel B) from suspended SAOS after incubation in the presence of mAb 90B8 in comparison with no antibody treatment or the control mAb W6/32.

DISCUSSION

In this study, we showed the induction of anoikis in SAOS cells after adhesion was denied on poly-HEMA treated cell culture wells. To our knowledge, these results are the first demonstration of anoikis in osteosarcoma cells. Based on the analyses of cell cycle and the results with caspase 3 and cytochrome c, it is fairly certain that the cell death seen in these studies is due to apoptosis and not necrosis. Under normal conditions cytochrome c resides in the space between the outer and inner membranes of mitochondria. During apoptosis cytochrome c translocates to the cytosol where it controls the assembly of an "apoptosome" composed of Apaf-1 and procaspase-9 [Li et al., 1997; Zou et al., 1999]. The formation of this complex results in the activation of the caspase cascade, which in turn orchestrates the events associated with apoptosis [Hengartner, 2000].

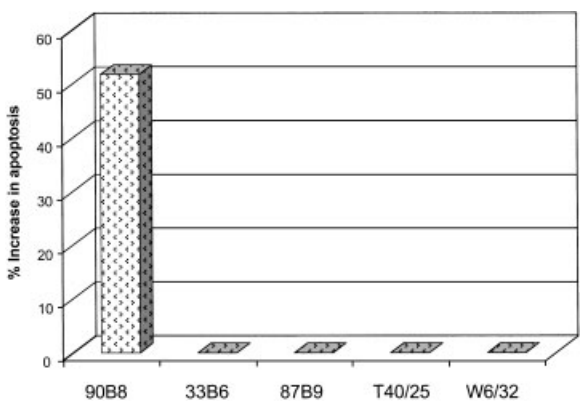

Fig. 6. Treatment of SAOS cells with anti-α4 mAb 90B8 increases anoikis of suspended cells. Suspended (poly-HEMA) SAOS cells were treated with the anti-α4 mAb 90B8, the anti-β1 mAbs 33B6 and 87B9, the anti-TCR mAb T40/25, or the anti-human MHC class I mAb W6/32 mAb for 24 h. Percent apoptosis was determined by flow cytometry analysis of DNA content after PI staining. Percentage increase in apoptosis was calculated as follows: (% apoptotic mAb-treated cells − % apoptotic untreated cells/% apoptotic untreated cells) × 100. Data shown are representative of three independent experiments.

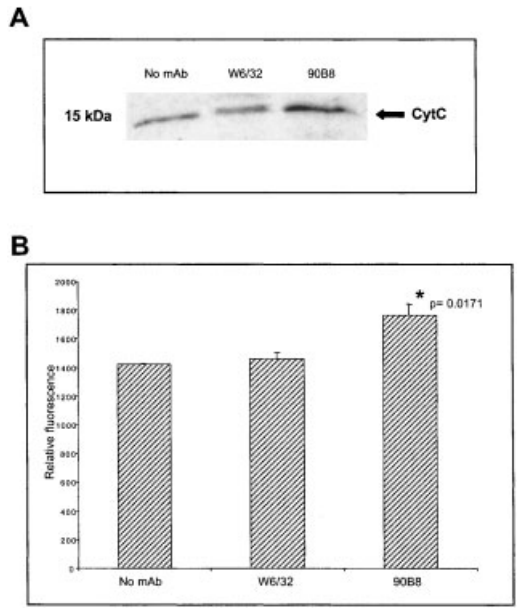

Fig. 7. Cytochrome c release and caspase 3 activity of suspended SAOS after treatment with anti-α4 mAb 90B8. SAOS cells suspended in poly-HEMA coated cell culture wells in the presence of anti-α4 mAb 90B8, anti-human class I mAb W6/32, or medium alone were assayed at 24 h for cytochrome c release (**A**) and caspase 3 activity (**B**).

In the analysis of integrin expression on SAOS cells, while there was low expression of α3β1, α5β1, and α6β1, the major β1 integrins expressed were α1β1 and α4β1. Recently, others have also shown that the integrin α4β1 is expressed on SAOS [Decker et al., 2002]. The integrin α4β1 was in primary osteosarcoma and metastatic osteosarcoma lesions of lung and pericardium. The expression of α4 integrins is in agreement with a previous report that identified α4 integrin in primary as well as metastatic pulmonary lesion using immunohistochemistry. In that study α4 integrin expression correlated well with the concomintant expression of its ligand, fibronectin, however, the other ligand for the alpha 4 integrin, VCAM-1, was not detected in their specimens [Kawaguchi and

Uede, 1993]. Interestingly, increased levels of α4 integrin have been found on metastatic sarcoma cells compared to primary sarcomas [Paavonen et al., 1994]. In contrast, expression of α4β1 heterodimers on the melanoma cell line B16 causes decreased matrigel invasion and suppressed pulmonary metastasis formation [Qian et al., 1994].

These observations led us to further examine the role of α4 integrin during anoikis of osteosarcoma cells. Our findings showed that culture of SAOS cells on poly-HEMA treated cell culture wells induces anoikis. Anoikis was further increased after the addition of the anti-α4 mAb 90B8. This result was somewhat surprising as it was predicted that antibody binding might send a survival signal that rescues the cells from anoikis. A previous study conducted in our laboratory has shown that the addition of anti-integrin mAbs rescues gastric adenocarcinoma cells from apoptosis despite suspended conditions [Caruso and McIntyre, 2001]. Other studies have shown that soluble collagen and fibronectin can also rescue cells from anoikis [Halvorson et al., 1998; Meerschaert et al., 1999]. In these cases mere ligation of integrins was enough to send the survival signal. In contrast, an integrin knockout model recently developed has shown that lack of αv integrins promotes cell survival and proliferation of endothelial cells resulting in enhanced angiogenesis. They also reported that the lack of integrin interaction, due to the absence of the ligand or by addition of an antagonist, causes cell death. The mechanism was associated with recruitment of caspase-8 to the plasma membrane by unligated integrins [Stupack et al., 2001; Cheresh and Stupack, 2002]. Finally, other investigators have shown that overexpression of integrins, such as α5β1, in intestinal epithelial cells is associated with reduced cell growth that results in protection against apoptosis [Lee and Juliano, 2000].

Our results differ from models whereby integrin ligation saves cells or lack of interaction induces cell death. We show an enhancement of cell death after integrin ligation with the anti-α4 mAb 90B8. We have not seen this augmentation with soluble ligands of the integrin α4β1 such as VCAM-1 (10 μg/ml) or the CS-1 region of fibronectin (10 μg/ml) (data not shown). The fact that the anti-α4 mAb 90B8 was able to further increase anoikis of SAOS cells suggest that a region of the α4 integrin subunit can be involved in the control of cell death. In support of this concept, it was reported that an anti-α4 antibody induces apoptosis in murine thymocytes [Tchilian et al., 1997]. In our study, anti-β1 integrin mAbs did not induce anoikis, suggesting that the α4 subunit could have a unique role in controlling cell death or that the binding of the anti-β1 mAb to α1β1 can overcome any apoptotic effect of the anti-β1 mAb binding to α4β1. Because the α4 and β1 subunits contain different regions associated with a variety of functions such as induction of adhesion or proliferation [Bednarczyk and McIntyre, 1990; Pulido et al., 1991; Bednarczyk et al., 1993, 1994; Teague and McIntyre, 1994], it is possible that unique sites on the α4 subunit may regulate anoikis. In support of this concept, we have also seen augmentation of anoikis with another anti-α4 mAb 91E12 but not with the anti-α4 mAb L25 or with a mAb 19H8 specific for a combinatorial α4β1 epitope (data not shown).

Induction of anoikis in suspended SAOS after treatment with the anti-α4 mAb 90B8 correlated with only a modest increase in cytochrome c release and caspase 3 activity. In Figure 5, when adherent cells were placed in suspension for 24 h, an 80% increase in caspase 3 activity was found. In Figure 7 when the anti-α4 mAb was added there was a 22% increase in caspase 3 activity over the no antibody control. It is not clear if this 22% difference accounts for all of the 52% increase in anoikis found under the same conditions. If not, other mechanisms may be involved, perhaps the activation of integrin-specific mediators such as FAK and subsequently Akt.

Our findings using osteosarcoma cells warrant additional studies to investigate the mechanisms of integrin activation of anoikis and the role of integrins in the pathogenesis of tumor metastasis. These studies may lead to the design of novel antibody and/or inhibitory peptide-based pharmacological approaches for the treatment of osteosarcoma.

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