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Characterization of Zinc-α_2-Glycoprotein as a Cell Adhesion Molecule That Inhibits the Proliferation of an Oral Tumor Cell Line

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Abstract Zn-α_2-glycoprotein (Zn$\alpha_2$gp) is a soluble protein widely distributed in body fluids and glandular epithelia. We have found it to be expressed in stratified epithelia as well. Zn$\alpha_2$gp is clinically correlated with differentiation in various epithelial tumors, including oral and epidermal tumors. We have cloned epidermal Zn$\alpha_2$gp and report the preparation of the recombinant protein in a Baculovirus expression system. Like the native molecule, recombinant Zn$\alpha_2$gp has RNase activity. Zn$\alpha_2$gp functions as a matrix protein for the Tu-138 oral squamous cell carcinoma cell line. Cell attachment to Zn$\alpha_2$gp is comparable to that for fibronectin and is inhibited by the synthetic RGD peptides RGD, RGDV, and RGDS. Attachment is also inhibited by the antibody to integrin $\alpha_5\beta_1$ (the fibronectin receptor), but not by antibodies to integrins $\alpha_v\beta_3$, $\alpha_3\beta_1$, and $\alpha_2\beta_1$. We find that the proliferation of Tu-138 cells is inhibited on a Zn$\alpha_2$gp matrix, as compared with other matrix proteins (fibronectin, vitronectin, laminin, and collagens I and IV) on which growth resembles that on the BSA control. We believe that the role of Zn$\alpha_2$gp in differentiation and its RNase activity are two likely suspects as agents of the inhibition of proliferation. J. Cell. Biochem. 75:160–169, 1999.

Key words: cell attachment; cell growth; cell-matrix interactions; integrins; cell-surface receptors

Zinc-α_2-glycoprotein (Zn$\alpha_2$gp) was initially purified from plasma [Bürgi and Schmid, 1961]; it can be precipitated by adding zinc ions and it displays electrophoretic mobility in the α_2-region of the plasma globulins, hence its name. It has been detected in most body fluids, including blood, seminal plasma, breast milk, synovial fluid, saliva, urine, and sweat [Poortmans and Schmid, 1968; Frenette et al., 1987; Ohkubo et al., 1990; Sánchez et al., 1992]. The antibody to this protein labels a wide variety of secretory epithelia in various human glands [Tada et al., 1991]. Northern blot analysis shows the gene to be expressed in liver, breast, prostate, kidney, pancreas, and several tumors [Schmid and Takahashi, 1964; Freije et al., 1991]. Zn$\alpha_2$gp has been cloned and the complete genomic sequence determined for prostate [Ueyama et al., 1993] and breast [Freije et al., 1993]. The cDNAs differ only in some minor substitutions and are ascribed to a single active gene and one or two pseudogenes. The corresponding amino acid sequences appear to be similar for these two tissues, as well as for blood plasma [Araki et al., 1988], consisting of a single polypeptide chain of 278 amino acids. The molecular weight is within the range 38–41 kDa, depending on the tissue; its fluctuation is associated with variations in glycosylation in different tissues, mostly about 12–18% carbohydrate, except that seminal plasma is unglycosylated [Ohkubo et al., 1990]. Direct sequenc-

Abbreviations used: BSA, bovine serum albumin; DMEM, Dulbecco's minimal essential medium; DPBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; RGD, Arg-Gly-Asp peptide sequence; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Zn$\alpha_2$gp, zinc-α_2-glycoprotein.

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ing of Zn$\alpha_2$gp is blocked at its N-amino acid terminus, pyroglutamic acid [Araki et al., 1988], again except in seminal plasma. We have found that Zn$\alpha_2$gp is also expressed in stratified epithelia; in particular, we have cloned it from epidermal keratinocytes and found a nucleotide sequence identical to that from prostate, differing only in posttranslational modifications [Lei et al., 1997].

Despite the widespread occurrence of Zn$\alpha_2$gp in different body tissues and the extensive studies of its molecular, protein, and crystalline structure [Sánchez et al., 1997], there are remarkably few clues to its function. Although Zn$\alpha_2$gp has considerable (36–39%) homology with major histocompatibility complex (MHC) class Iα chains in its amino acid sequence and domain structure [Araki et al., 1988], the consequent suspicion that it plays an immunological role has not been substantiated. Zn$\alpha_2$gp has been used extensively as a clinical marker of the histological grade of breast cancer tumors [Díez-Itza et al., 1993] and in prostate cancer [Frenette et al., 1987]. Higher levels of Zn$\alpha_2$gp are observed in the better differentiated cells, but without mechanistic explanation. We have confirmed these trends for normal epidermal and oral epithelia [Brysk et al., 1997a] and for oral squamous cell carcinomas [Brysk et al., 1999]. We have shown that Zn$\alpha_2$gp has ribonuclease activity against single-stranded RNA and preferentially cleaves RNA at its pyrimidine residues [Lei et al., 1998]. A human renal cell carcinoma line was found to attach and spread on a coating of Zn$\alpha_2$gp, while melanoma, breast carcinoma, prostatic adenocarcinoma, and osteosarcoma cell lines exhibited little or no binding [Takagaki et al., 1994]. We have investigated the matrix protein properties of Zn$\alpha_2$gp on the Tu-138 oral squamous cell carcinoma cell line. We report the preparation of recombinant Zn$\alpha_2$gp, its effect on the adhesion and proliferation of the cells (as compared with other matrix proteins), and the determination of the integrin receptor for Zn$\alpha_2$gp.

MATERIALS AND METHODS

Preparation of Recombinant Zn$\alpha_2$gp in a Baculovirus Expression System

Full-length Zn$\alpha_2$gp cDNA was cloned from our epidermal keratinocyte cDNA library [Lei et al., 1997]. To obtain restriction enzyme cuts compatible with the multiple cloning site of the Baculovirus expression vector PVL 1392, at each flank of the Zn$\alpha_2$gp cDNA, the cDNA was amplified by polymerase chain reaction (PCR) using two primers for the sense and antisense oligonucleotides: 5′-GATCAGATCTGCAGCAAGAATGGTGCCTGTC-3′ and 5′-CCTTCTAGAAAGCTAGGCAAGGAGGGATG-3′.

PCR was performed for 30 cycles, each consisting of a denaturation step at 94°C for 1 min, a reannealing at 60°C for 1 min, and an extension at 72°C for 2 min. The PCR products were fractionated on a 1% agarose gel and further purified using the QIAquick Gel Extraction kit (QIAGEN, Valencia, CA). The pure cDNA was doubly digested with the restriction enzymes *Bgl*II and *Xba*I, then inserted into the vector PVL1392 previously digested with the same enzymes downstream of the polyhefrin promoter. The vector PVL1392, containing the Zn$\alpha_2$gp cDNA, was subcloned into DH5α competent cells (Life Technologies, Gaithersburg, MD) according to the procedure recommended by the manufacturer. In order to generate the recombinant Baculovirus, 2 µg of PVL1392 vector, containing the full-length Zn$\alpha_2$gp cDNA, was mixed with 0.2 µg of BaculoGold Baculovirus DNA in the presence of 0.75 ml of the transfection buffer A (PharMingen, San Diego, CA). The transfection mixture was added dropwise to a monolayer of Sf 9 insect cells cultured in the TMN-FH insect medium (PharMingen). The cells were kept at 27°C for 4 h; the medium was then removed from the co-transfection plate. The cells were washed twice with 3 ml of fresh TMN-FH medium and maintained in the fresh medium for 3–5 days before harvesting. A time-course study was conducted at 36, 48, 60, 72, and 80 h postinfection, to assess optimal protein production. The cells were pelleted by centrifugation at 1,200*g* for 10 min, resuspended in 10 mM TE buffer (pH 8.0), and then centrifuged at 2,500*g* for 15 min. The supernatant containing the recombinant Zn$\alpha_2$gp was further centrifuged at 4,000*g* for 15 min at 4°C.

Purification of Recombinant Zn$\alpha_2$gp

The purification procedure for Zn$\alpha_2$gp was previously described by us [Lei et al., 1997]. Briefly, the extract containing recombinant Zn$\alpha_2$gp was dialyzed against water for 16 h at 4°C. The extract was then poured into a slurry of Con A-Sepharose in a buffer of 10 mM HEPES, pH, 8.0, containing 0.5 mM $MnCl_2$, 0.5 mM $CaCl_2$, 0.5 mM NaCl, and 0.1% NaN_3, then gently rotated on a nutator for 16 h at 4°C. The

unbound proteins were removed by extensively washing the sepharose gel with the Con A buffer, and the absorbed glycoproteins were eluted with 1 M α-methyl-D-mannoside. The eluent was first dialyzed overnight at 4°C against Tris-acetate buffer, then concentrated in a Filtron Omega cell unit (22 µm). The glycoproteins were further fractioned by gel-filtration high-performance liquid chromatography (HPLC) on a G-3000 SWDX high-resolution HPLC analytical column. The 35- to 45-kDa fractions containing Zn$\alpha_2$gp were then pooled, concentrated, and stored in 100 mM Tris-HCl, pH 7.4. Native Zn$\alpha_2$gp was isolated from human epidermis as previously described [Lei et al., 1997] and then purified by the same procedures as the recombinant molecule. Zn$\alpha_2$gp antiserum and prostate Zn$\alpha_2$gp were gifts from Dr. I. Ohkubo.

Western Blotting

Prostate Zn$\alpha_2$gp and epidermal native and recombinant Zn$\alpha_2$gp were analyzed by electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and stained with Coomassie blue. A duplicate gel was electrophoretically transferred to a nitrocellulose membrane in a buffer of 20% methanol, 25 mM Tris-HCl, and 192 mM glycine. The blots were quenched in TBST (10 mM Tris-HCl, pH 8.8, 150 mM NaCl, 0.05% Tween 20), containing 3% nonfat dried milk, for 30 min and then washed twice for 10 min in the buffer alone. The blot was then incubated for 1 h with a 1:500 dilution of rabbit anti-prostate Zn$\alpha_2$gp IgG in 0.5% dried milk in TBST and then washed twice for 10 min each with unsupplemented TBST. The blot was then incubated for 1 h in a 1:500 dilution of peroxidase-conjugated sheep anti-rabbit IgG (Cappel, ICN Biomedicals, Costa Mesa, CA), and the color was developed by the ECL (Enhanced Chemiluminescence) kit (Amersham Life Science, Arlington Heights, IL).

Zymography

Samples of prostate Zn$\alpha_2$gp and epidermal native and recombinant Zn$\alpha_2$gp were separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (20 µg/lane). The RNase activities were assayed by zymography, by a slight modification of a published protocol [Bravo et al., 1994]. Briefly, human epidermal total RNA, together with 2 mM EDTA, was added to the SDS-PAGE separating gel. After electrophoresis, the gel was washed twice for 20 min in 10 mM Tris-HCl, pH 8.0, also containing 20% isopropanol, under gentle shaking, followed by two 20-min washes in 10 mM Tris-HCl, pH 8.0, all at room temperature. The gel was then incubated in a buffer of 100 mM Tris-HCl, pH 8.0, and stained for 5 min with 0.5 µg/ml ethidium bromide. The bands were visualized under ultraviolet (UV) light and photographed.

Cell Culture

We used the Tu-138 squamous cell carcinoma cell line, derived from a gingival tumor [Liu et al., 1994]. It was grown and maintained in Dulbecco's minimal essential medium (DMEM)-F12 (1:1) supplemented with 10% fetal bovine serum (FBS), penicilli,n and streptomycin. Tissue culture media and FBS were obtained from Gibco-BRL Life Technologies (Grand Island, NY).

Cell Attachment and Spreading

Ninety-six-well plates were coated overnight at 4°C with dilutions (10–50 µg/ml) of individual matrix proteins: Matrigel, collagen IV (Collaborative Research, Bedford, MA); laminin, vitronectin (Chemicon, Temecula, CA); fibronectin, collagen I (Sigma Chemical Co., St. Louis, MO); and recombinant Zn$\alpha_2$gp. The wells were blocked by incubation for 30 min with 3% heat-denatured bovine serum albumin (BSA) in Dulbecco's phosphate-buffered saline (DPBS), then rinsed 3 times with DPBS. Cultured Tu-138 cells were detached from the culture vessels by incubation with a solution of 0.025% trypsin-0.025% EDTA in DPBS, washed in culture medium containing 10% FBS, and then in the medium alone without serum. The cells were counted in a hematocytometer and adjusted to a concentration of 1×10^5 cells per ml. Aliquots (100 µl) of the cells suspension were seeded onto the matrix-coated wells of the plate and allowed to attach for 2 h at 37°C in a 5% CO_2 incubator. Unbound cells were removed by gently washing the wells four times with warm DMEM, rinsed with DPBS, and assessed by the MTT assay (described below). Selected wells were photographed under a phase-contrast microscope to measure cell spreading.

MTT Assay of Cell Growth

Cell numbers in 96-well plates were determined by the MTT assay [Takagaki et al., 1994].

After aspiration of nonattached cells and culture medium, the wells were filled with 100 µl of serum-free medium containing 25 µl (5 mg/ml in DPBS) of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) and incubated for 4 h at 37°C. The insoluble dye product was solubilized overnight at 37°C with 10% SDS containing 0.01 N HCl. Conversion of MTT to the formazan by metabolically viable cells was measured at 450 nm in a microplate reader (THERMA MAX, Molecular Devices, Sunnyvale, CA). Experiments were performed in triplicate.

Assay of Inhibition of Cell Attachment

Ninety-six-well plates were coated with Zn$\alpha_2$gp (at 20 µg/ml) as described above. We used the RGD, RGDS, and REDV synthetic peptides (Bachem, Torrance, CA) to assess inhibition of cell attachment to recombinant Zn$\alpha_2$gp. We also used antibodies against $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_3\beta_1$, and $\alpha_2\beta_1$ to assess which integrins were involved in cell adhesion to Zn$\alpha_2$gp. Cell numbers were determined by the MTT assay in triplicate wells.

Effect of Zn$\alpha_2$gp on Cell Proliferation

Ninety-six-well plates were coated with 20 µg/ml of Zn$\alpha_2$gp, fibronectin, vitronectin, laminin, collagen I, collagen IV, and BSA, as described above. Tu-138 cells were adjusted to a concentration of 1×10^5 cells per ml, and aliquots (100 µl) of the cell suspension were seeded onto the matrix-coated wells. The cells were allowed to attach for 4 h at 37°C in a 5% CO_2 incubator. Unattached cells were removed, and complete medium containing 10% FBS was then added. Cell growth was measured daily for 4 days by the MTT assay in triplicate wells.

RESULTS

Preparation and Purification of Recombinant Zn$\alpha_2$gp

We prepared recombinant Zn$\alpha_2$gp in a Baculovirus expression system. Zn$\alpha_2$gp was easily purified from the crude insect extract by Con A affinity chromatography and gel filtration. Figure 1 compares the following on SDS-PAGE: (Fig. 1A) prostate Zn$\alpha_2$gp, (Fig. 1B) native epidermal Zn$\alpha_2$gp, and (Fig. 1C) recombinant epidermal Zn$\alpha_2$gp. Lanes A1, B1, and C1 display SDS-PAGE gels stained with Coomassie blue, showing the migration of each variety of Zn$\alpha_2$gp.

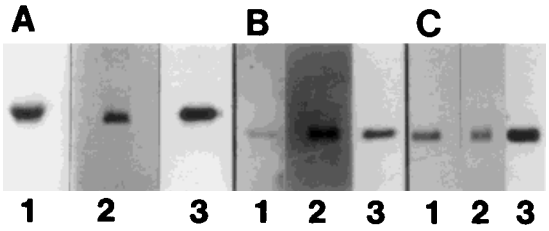

Fig. 1. Identification of native Zn$\alpha_2$gp extracted from **(A)** prostate and **(B)** epidermis, as well as of **(C)** recombinant epidermal Zn$\alpha_2$gp. **Lane 1**, Coomassie-stained gels; the prostate Zn$\alpha_2$gp migrated at 38 kDa, while that from the epidermis was at 34 kDa; **lane 2**, RNase activities demonstrated by zymography; **lane 3**, corresponding Western blots.

Epidermal Zn$\alpha_2$gp, both native and recombinant, has a slightly lower molecular weight (34 kDa) than does prostate Zn$\alpha_2$gp (38 kDa); the discrepancy is probably due to posttranslational modifications. To confirm the identification, we include a Western blot probed with antiserum to prostate Zn$\alpha_2$gp (Fig. 1 A3, B3, C3); all three molecules strongly react with the antiserum. We have previously reported that both epidermal and prostate Zn$\alpha_2$gp have ribonuclease activity [Lei et al., 1998]; so does the recombinant protein, as shown by zymography, upon including RNA in the SDS-PAGE running gel (Fig. 1 A2, B2, C2).

We had previously shown by amino acid sequencing that native epidermal Zn$\alpha_2$gp has pyroglutamic acid as the N-terminal [Lei et al., 1998], like plasma Zn$\alpha_2$gp [Araki et al., 1988]. We have verified that recombinant Zn$\alpha_2$gp has the same N-terminus.

Cell Adhesion to Zn$\alpha_2$gp

Zn$\alpha_2$gp contains an RGD (Arg-Gly-Asp) sequence at amino acid residues 231–233. RGD sequences are characteristic of many matrix proteins. We assessed whether Zn$\alpha_2$gp was an adhesion molecule, using Tu-138 oral epithelial tumor cells. Figure 2 displays the results by phase-contrast microscopy. The least cell spreading occurred on a matrix of BSA (negative control) and most cell spreading on a matrix of Matrigel (positive control). Figure 3 displays the results quantitatively by the MTT assay. Cell binding and spreading was about equal on Zn$\alpha_2$gp and on fibronectin, and progressively lower on laminin, vitronectin, and collagens I and IV.

Furthermore, the cells bind and spread on Zn$\alpha_2$gp in a dose-dependent fashion, as can be seen by phase-contrast microscopy (Fig. 4) and

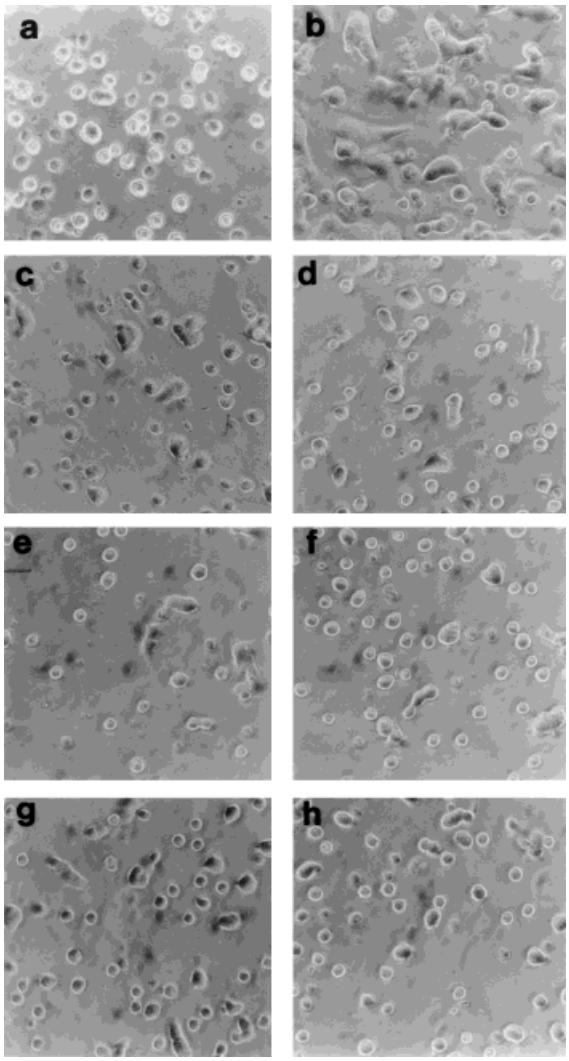

Fig. 2. Phase-contrast micrographs of Tu-138 oral carcinoma cells attached to different matrix proteins: **(a)** BSA (as control), **(b)** Matrigel, **(c)** Zn$\alpha_2$gp, **(d)** fibronectin, **(e)** vitronectin, **(f)** laminin, **(g)** collagen I, and **(h)** collagen IV.

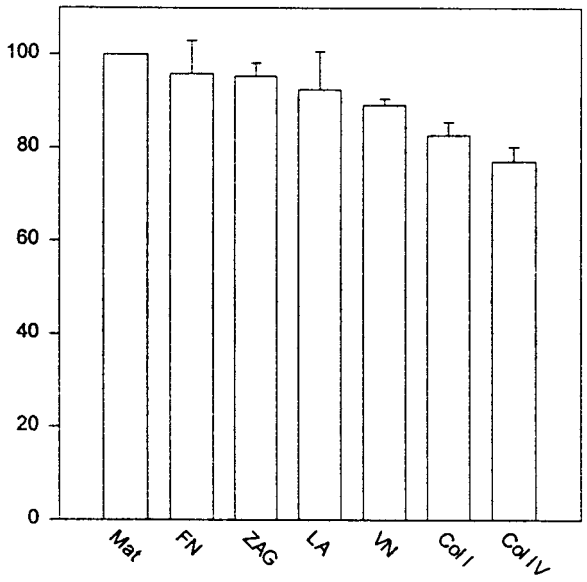

Fig. 3. Attachment of Tu-138 cells to different matrix proteins (10 µg/ml), by the MTT assay, as percentage of binding to Matrigel.

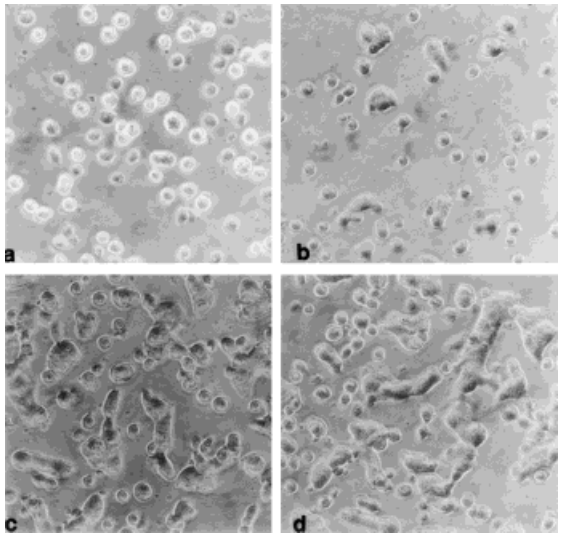

Fig. 4. Phase-contrast micrographs of cell attachment to different concentrations of Zn$\alpha_2$gp: **(a)** BSA control, **(b)** 10, **(c)** 20, and (**d)** 40 µg/ml of Zn$\alpha_2$gp.

quantitatively by the MTT assay (Fig. 5). Attachment and spreading increased linearly within the range 2–10 µg/ml of Zn$\alpha_2$gp and peaked at 20 µg/ml.

Inhibition by RGD Peptides

To demonstrate that the cell binding was specific for the RGD sequence, we ran competitive inhibition assays with the inclusion of RGD peptides. Cell attachment to Zn$\alpha_2$gp was inhibited by the synthetic peptides RGD, RGDS, and RGDV, in a dose-dependent manner, with optimal inhibition at about 0.2 µg/ml of each (Fig. 6). The inhibition was only partial, however, remaining less than a factor of 2 asymptotically; this finding suggests that there must be other binding sites of consequence.

Inhibition by Antibodies to Specific Integrins

We show by SDS-PAGE and ECL that Tu-138 cells express the integrin subunits β_1, β_3, α_5, and α_v (Fig. 7). To determine which integrins are involved in cell adhesion to Zn$\alpha_2$gp, we performed competitive inhibition assays using antibodies against specific integrins. Antibody

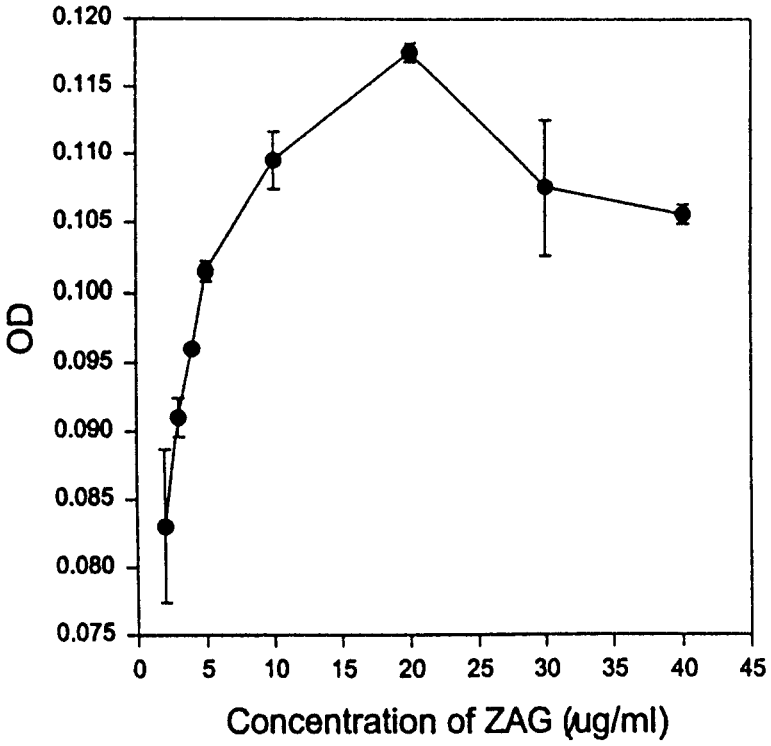

Fig. 5. Dose response of cell attachment to Zn$\alpha_2$gp, by the MTT assay.

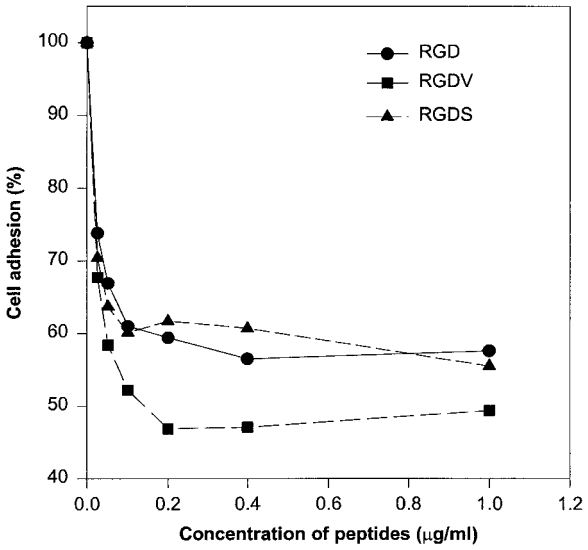

Fig. 6. Inhibition by RGD peptides of cell attachment to Zn$\alpha_2$gp (dose response).

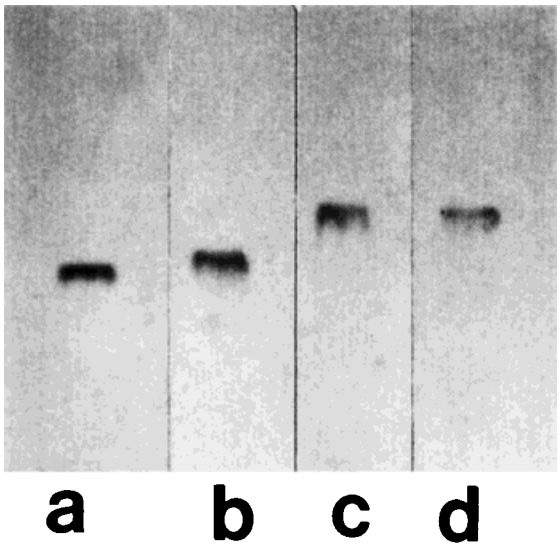

Fig. 7. Integrin expression in Tu-138 cells, as demonstrated by enhanced chemiluminescence, using antibodies to the subunits **(a)** β_1; **(b)** β_3; **(c)** α_5; and **(d)** α_v.

against $\alpha_5\beta_1$ strongly inhibited cell binding; antibody against $\alpha_v\beta_3$ showed a slight inhibition, while those against $\alpha_3\beta_1$ and $\alpha_2\beta_1$ were ineffective (Fig. 8).

Cell Proliferation on a Zn$\alpha_2$gp Matrix

We also investigated whether the proliferation of Tu-138 cells was affected by growing the cells for four days on different matrix proteins, including Zn$\alpha_2$gp. Figure 9 measures cell growth by the MTT assay. We find slight growth inhibition, and only on day 4, for the matrix proteins fibronectin, vitronectin, laminin, and collagens I and IV. On the other hand, cells grown on a Zn$\alpha_2$gp matrix are totally growth-inhibited on

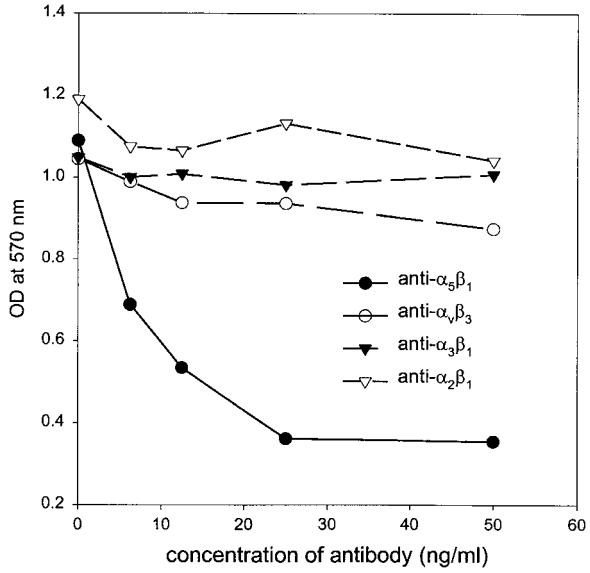

Fig. 8. Inhibition of cell attachment to Zn$\alpha_2$gp by antibodies to different integrins.

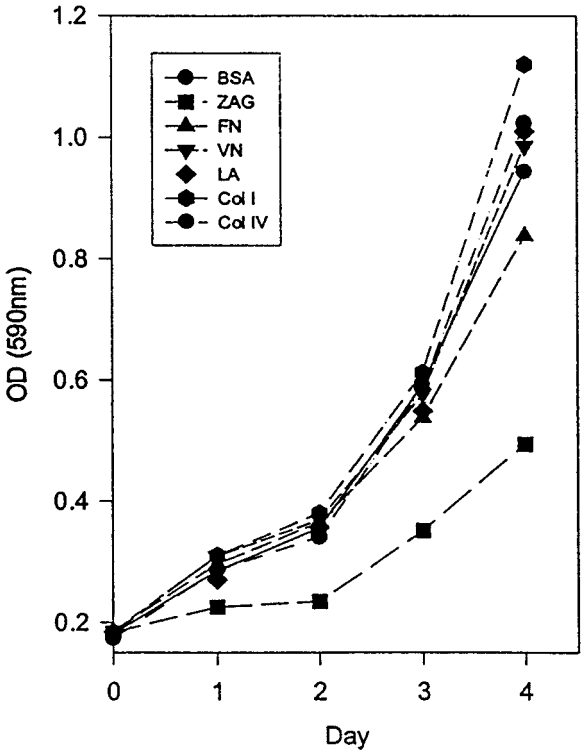

Fig. 9. Comparison of growth of Tu-138 cells on different matrix proteins, demonstrating inhibition by Zn$\alpha_2$gp, but not by the others.

days 1 and 2; they resume their proliferation on days 3 and 4, but at a reduced rate. The cells grown on Zn$\alpha_2$gp were viable by trypan blue exclusion.

DISCUSSION

We have shown that Zn$\alpha_2$gp functions as a matrix protein for the Tu-138 human oral epithelial squamous cell carcinoma cell line. Adhesion of the cells to Zn$\alpha_2$gp and to fibronectin was comparable, while binding to laminin, vitronectin, collagen I, and collagen IV was progressively lower. Binding and spreading of the cells on a Zn$\alpha_2$gp matrix occurred in a dose-dependent manner. Attachment to a matrix of Zn$\alpha_2$gp was previously reported for the SMKT R-3 cell line [Takagaki et al., 1994]. Adhesion of these human epithelial renal carcinoma cells [Miyao et al., 1989] was greatest on fibronectin, then progressively lower on laminin, vitronectin, Zn$\alpha_2$gp, and collagen IV; the order is similar to what we found for the Tu-138 cells, except that Zn$\alpha_2$gp ranks considerably lower in binding for the SMKT R-3 cells relative to the other matrix proteins.

Tu-138 cells grew on matrices of fibronectin, laminin, vitronectin, and collagens I and IV at about the same rate as on the BSA control. With Tu-138 cells plated on a Zn$\alpha_2$gp matrix, there was a total inhibition of growth for the first 2 days; thereafter, proliferation resumed but at a lower rate. This is the first published observation of an impact of Zn$\alpha_2$gp on cell proliferation.

Inhibition of proliferation is often associated with the induction of differentiation, particularly for epithelial cells, but also for adenocarcinomas and others. Zn$\alpha_2$gp expression has been correlated with differentiation in various cell types (including those that bind poorly to a Zn$\alpha_2$gp matrix). A significant association has been observed between Zn$\alpha_2$gp levels and the histological grade of breast cancer tumors, with higher levels found in well-differentiated tumors than in poorly differentiated tumors, by protein assays [Díez-Itza et al., 1993], as well as at the mRNA level [Freije et al., 1991]. Zn$\alpha_2$gp mRNA expression is lower in oral squamous cell carcinomas than in normal tissue; among the tumors, it is highest in the better differentiated tumors [Brysk et al., 1999]. Analogously, Zn$\alpha_2$gp levels are much higher in benign prostatic hyperplasia than in adenocarcinoma of the prostate [Frenette et al., 1987]. We have found that Zn$\alpha_2$gp gene expression is also associated with differentiation in epidermal tumors (with higher levels in normal epithelia than in

squamous cell carcinomas or Merkel cell carcinomas, and higher in the latter than in basal cell carcinomas) [Lei et al., 1997]. In addition, the gene expression of Zn$\alpha_2$gp in tumor cell lines is upregulated by agents that induce differentiation, such as interferon-γ [Brysk et al., 1997b] and glucocorticoids [López-Boado et al., 1994].

Matrix proteins can regulate both proliferation and differentiation. Ligation of integrin $\alpha_5\beta_1$ with fibronectin inhibits growth in HT29 colon carcinoma cells [Varner et al., 1995], although the mechanism is undetermined. There is a similar effect of Zn$\alpha_2$gp in Tu-138 cells. Signal transduction pathways become activated when specific matrix proteins interact with their cell surface integrin receptors. While cessation of epithelial cell growth is ordinarily accompanied by an increase in differentiation, the two processes can be uncoupled. In epidermal cells, antisense c-*myc* oligomer inhibits growth without inducing differentiation [Hashiro et al., 1991]. Transforming growth factor (TGF-β1) induces reversible growth arrest of epidermal keratinocytes by inducing c-*myc*, without impacting cellular maturation [Pietenpol et al., 1990].

Another mechanism by which Zn$\alpha_2$gp may impact proliferation is our finding that it has ribonuclease activity [Lei et al., 1998]. Some RNases have been used as antitumor agents because they inhibit proliferation. In particular, bovine seminal RNase and frog egg RNase (onconase) have been found to be cytotoxic to tumor cells in vivo and in vitro [Laccetti et al., 1992]. RNase L has been implicated in growth arrest and maturation in embryonal carcinoma cell lines; the carcinoma clones are inhibited preferentially with differentiation [Krause et al., 1985; Silverman, 1994]. The specific RNase activity of Zn$\alpha_2$gp is comparable to that of onconase. Zn$\alpha_2$gp preferentially cleaves ssRNA substrates and is pyrimidine-specific [Lei et al., 1998], like onconase [Wu et al., 1993]. The N-terminus of both is pyroglutamic acid, which is also the site of the RNase activity of onconase [Wu et al., 1993]. The antiproliferative activity of onconase has been linked to its insensitivity to RNase inhibitors, in contrast to RNase A, which is extremely sensitive to RNase inhibitors and does not exhibit antitumor activity [Wu et al., 1993]. The fact that Zn$\alpha_2$gp is only partially inhibited by placental RNase inhibitor suggests that it may, like onconase, escape being neutralized by endogenous inhibitors.

The cell surface receptors of matrix proteins are usually integrins. Combinations of cell-surface integrins allow cells to adhere to, and migrate on, a variety of matrix proteins. Integrins regulate many biological processes, including motility, proliferation, differentiation, and apoptosis [Bates et al., 1995; Ruoslahti, 1996; Zhang et al., 1995; Clarke et al., 1995]. Integrins are transmembrane heterodimers composed of associated α- and β-subunits, the particular combinations of which determine ligand specificity. We found, by ECL, that Tu-138 cells express the integrin subunits α_5, α_v, β_1, and β_3. Using antibodies to specific integrins, we showed that cell binding to Zn$\alpha_2$gp was inhibited by an antibody to integrin $\alpha_5\beta_1$, but not by antibodies to integrins $\alpha_v\beta_3$, $\alpha_3\beta_1$, and $\alpha_2\beta_1$.

Many of the β-subunits recognize the tripeptide Arg-Gly-Asp (RGD) in their ligands, which is characteristic of most matrix proteins. Interactions between tumor cells and their substratum play an important role in neoplastic progression. The anchorage-independent growth of tumor cells may be a consequence of transformation-associated changes in intracellular signaling that disrupt integrin-mediated attachment to the substratum [Dedhar, 1995; Ruoslahti, 1996]. Many tumor cells lose their ability to attach to fibronectin after transformation [Wagner et al., 1981; Ruoslahti, 1996]. This may be the result of loss of the fibronectin receptor, integrin $\alpha_5\beta_1$; when $\alpha_5\beta_1$ was transfected into colon tumor cells, which lack the receptor, the cells regained their ability to bind to fibronectin [Varner et al, 1995]. The Tu-138 oral tumor cell line, in contrast to the colon tumor cells, retains its fibronectin receptor. Like fibronectin, Zn$\alpha_2$gp contains an RGD peptide sequence. It also has REDI and ILDR sequences similar to the REDV and ILDV cell-recognition regions of fibronectin. Attachment of the Tu-138 cells to Zn$\alpha_2$gp was inhibited in a dose-dependent manner by synthetic RGD peptides, by up to about a factor of 2, as had also been found for the SMKT R-3 cells. The incomplete inhibition may be due to residual alternative binding, perhaps to REDI and ILDR sequences in Zn$\alpha_2$gp, or may reflect multiple integrin receptors recognizing identical ligands.

The similarities between Zn$\alpha_2$gp and fibronectin extend to their integrin receptors: both bind preferentially to integrin $\alpha_5\beta_1$. Despite the com-

mon integrin receptor, however, they are associated with divergent and often opposite functions. Fibronectin inhibits terminal differentiation in epidermal keratinocytes [Adams and Watt, 1989]. On the contrary, Zn$\alpha_2$gp expression is higher in well-differentiated cells and decreases with tumor dedifferentiation, as detailed above. The expression of fibronectin increases with tumor progression in some tumors [Sheibani et al., 1991] but decreases in others [Wagner et al., 1981; Ruoslahti, 1996]. Differences in cell binding to fibronectin with tumor progression reflect not only the expression of fibronectin, but also the expression of the fibronectin receptor. When integrin α_5 was transfected into tumor cells not normally containing it, the cells regained their ability to bind to fibronectin [Varner et al., 1995] and the transfectants had an increased rate of proliferation when grown on fibronectin. By contrast, we have found that Zn$\alpha_2$gp inhibits proliferation of the Tu-138 cells. These cells express integrin α_5, and their proliferation is about the same on a matrix of fibronectin as on the BSA control. Fibronectin and Zn$\alpha_2$gp are both widespread and abundant endogenous proteins. Their conflicting properties lend interest to their competition for the same integrin receptor.

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