Cell adhesion to low- M_r proteins extractable from mineralized and soft connective tissues

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Guanidinium chloride (4 M) containing proteinase inhibitors was used to extract proteins from porcine calvariae and long bones. The extracted proteins were separated on polyacrylamide slab gels and transferred electrophoretically to nitrocellulose strips. Proteins with cell-adhesion properties were identified by incubating the strips with cells and staining with Amido Black. In addition to binding to fibronectin, both bone cells and fibroblast-like cells adhered to proteins of $M_r \sim 30000$ and ~ 14000 – 17000 . 4 M-Guanidinium chloride extracts of porcine skin and gingiva yielded cell-binding proteins with similar M_r values. These data suggest that these low- M_r proteins may have a general cell-adhesion function in both soft and mineralized connective tissues.

INTRODUCTION

Cellular adhesiveness is a property fundamental to many biological processes, including normal cell growth, tissue morphogenesis, wound healing, and tumour metastasis (for recent reviews, see Yamada [1], Grinnell [2], Hay [3] and Kleinman *et al.* [4]). The ability of cells to adhere to various substrata is mediated by specific molecules at the cell surface and in the ECM. A number of different proteins have been identified that facilitate cellular attachment. For example, the ability of fibronectin (M_r 440000–500000) to mediate the attachment of a wide variety of cells to collagen, fibrin and artificial tissue-culture plastics has been characterized extensively (reviews cited above). Laminin is a second well-characterized adhesive glycoprotein (M_r 1000000) that appears to be localized exclusively in basement membranes and, although not strictly specific for epithelial cells, does mediate their attachment to type IV collagen [5, 6] (for reviews see Yamada [1] and Hakomori *et al.* [7]). A third glycoprotein, serum spreading factor or vitronectin (M_r 70000), exists in both serum and tissues and promotes the attachment and spreading of both fibroblastic and epithelial cells *in vitro* [8–12]. Another monomeric serum protein with a similar size (M_r 62000–70000) is a spreading factor for both fibroblastic and epithelial cells [13–15]. The possibility of any structural relationships between these proteins remains to be elucidated. In addition to these proteins, a small number of other macromolecules have been isolated and partially characterized that may mediate the attachment of specific cell populations to certain, or specialized, substrata. For example, chondronectin (M_r 180000), a glycoprotein isolated from chicken serum, appears to be specific for promoting the attachment of chondrocytes to type II collagen *in vitro*, and displays minimal attachment activity for fibroblasts or epithelial cells [16, 17]. Another serum glycoprotein (M_r 65000) that promotes the spreading of epithelial cells, termed epibolin, has been described [18]. Whether it is strictly specific for epithelial cells and/or is related to any of the other serum spreading

factors outlined above needs to be determined. Another protein, epinectin (M_r 70000), which is different from epibolin and mediates the attachment of A431 cells from whose extracellular matrix the protein was extracted, may be localized specifically in squamous epithelium *in situ*, but has yet to be tested for its ability to mediate the attachment of other cell types [19].

We have initiated an investigation of cell attachment to bone-derived macromolecules, since serum and fibronectin-depleted serum as well as fibronectin increase the attachment of rat calvaria cells to type I collagen [20, 21], suggesting that molecules other than fibronectin can specifically promote bone-cell attachment. By using the cell-attachment assay on replicas of SDS/polyacrylamide gels devised by Hayman *et al.* [11], we have detected cell-attachment molecules, not previously identified, corresponding to a number of discrete M_r ranges.

MATERIALS AND METHODS

Cell culture

Rat calvaria bone cells [22] and porcine periodontal ligament fibroblasts [23] were isolated and characterized as described in the references cited. RCB 2.2 cells are a clonally derived osteoblast-like population from rat calvariae [22]. For routine maintenance, cells were cultured in plastic tissue-culture flasks (Flow Laboratories, Rockville, MD, U.S.A.) in α -MEM supplemented with 15% (v/v) FBS (Gibco, Grand Island, NY, U.S.A.) and antibiotics (100 μ g of penicillin G, 50 μ g of gentamycin and 300 ng of amphotericin B/ml), at 37 °C in humidified air/CO₂ (19:1). For subculture, cells were treated with 0.01% (w/v) trypsin in citrate/saline (4.4 g of trisodium citrate dihydrate and 10 g of KCl/litre, pH 7.2).

Tissue preparation and extraction

Proteins were isolated from various connective tissues by using the dissociative extracting conditions described by Butler *et al.* [24]. Porcine long bones and calvariae were

Abbreviations used: GdmCl, guanidinium chloride; SDS, sodium dodecyl sulphate; α -MEM, alpha minimal essential medium; FBS, foetal-bovine serum; ECM, extracellular matrix; PMSF, phenylmethanesulphonyl fluoride; phosphate-buffered saline, 8.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na₂HPO₄, 0.2 g of KH₂PO₄, 0.13 g of CaCl₂·2H₂O and 0.1 g of MgCl₂·6H₂O/litre, pH 7.3.

collected within 3 h of death of the animals. The bones were cleaned free of soft tissues, washed three times in cold phosphate-buffered saline in the presence of proteinase inhibitors (0.05 M-6-aminohexanoic acid, 0.05 M-benzamidine and 0.001 M-PMSF), and finally washed with the above solution overnight at 4 °C. This mixture was then filtered and the bone portion was dissociatively extracted with 4 M-GdmCl in 0.05 M-Tris/HCl, pH 7.4, and proteinase inhibitors at a volume of 200 ml of extractant/g of bone. This extraction was carried out for 4 days at 4 °C; the samples were then filtered and the solutions were concentrated by ultra-filtration on an Amicon PM 10 membrane. The concentrated extract was then dialysed against 0.5 M-sodium acetate, pH 7.4 (two changes), followed by water (three changes). The protein concentrations were determined by the Bio-Rad protein assay, which is based on the Bradford [25] method. For preparation of ECM material from RCB 2.2 cells, cells were grown to confluence in 100 mm-diameter plastic tissue-culture dishes in the medium described above. Medium was removed, cells were washed with three changes of phosphate-buffered saline and extracted with 4 M-GdmCl as for tissues.

Electrophoresis and transfer of proteins

Proteins were separated on gradient slab gels of 5–20% polyacrylamide in the presence of SDS and 2-mercaptoethanol. M_r standards were: myosin, 200 000; β -galactosidase, 116 500; phosphorylase *b*, 92 000; bovine serum albumin, 66 200; ovalbumin, 45 000; carbonic anhydrase, 31 000; soya-bean trypsin inhibitor, 21 500; and lysozyme, 14 400 (Bio-Rad electrophoresis standards; Bio-Rad Laboratories, Richmond, CA, U.S.A.). Human plasma fibronectin was obtained from Collaborative Research, Lexington, MA, U.S.A. Proteins were transferred electrophoretically to nitrocellulose as described by Towbin *et al.* [26] and protein bands were detected by staining with Amido Black.

Cell attachment to proteins on nitrocellulose filters

Cell binding to proteins on nitrocellulose filters was done by modification of the procedure of Hayman *et al.* [11]. After transfer of the proteins to the membrane strips, the strips were incubated for 1 h at room temperature with a 3% (w/v) solution of bovine serum albumin in phosphate-buffered saline, followed by three 15 min washes in phosphate-buffered saline. Subconfluent exponential-phase cells were prepared for binding assays by trypsin treatment and washing them in phosphate-buffered saline (three changes), resuspending in serum-free α -MEM and incubating at 37 °C for 1 h. After this recovery time, cells were counted in a Coulter counter and the cell number was adjusted to 2×10^5 cells/ml. They were then added gently to the bovine serum albumin-treated nitrocellulose strips in aluminium foil 'boats', to a final concentration of 5×10^6 – 1×10^7 cells/strip, and incubated for 2 h at 37 °C with intermittent shaking to disperse clumps and resuspend the non-attached cells. The strips were then washed in phosphate-buffered saline (two changes), and the attached cells were fixed with 3.7% (v/v) formaldehyde in phosphate-buffered saline, followed by washing in phosphate-buffered saline (three changes). The cell-binding regions were detected by staining with Amido Black. It should be noted that, on bovine serum albumin-treated nitrocellulose strips stain-

ed with Amido Black, transferred protein bands are normally not detectable, since the strip stains an overall pale blue because of the bovine serum albumin coating. Cells, however, label very dark indigo, and protein bands with cell-binding capacity are detectable as very dark indigo bands against the pale-blue background.

Cell binding to proteins on nitrocellulose strips was not observed at 4 °C.

RESULTS

The most prominent protein bands from porcine long bones and calvariae were at M_r ranges of 220 000 (co-migrating with human plasma fibronectin; Fig. 1, lane 3), 125 000 [co-migrating with type I collagen α -chains (not shown)], 45 000–50 000, approx. 30 000, and a series of very strongly staining bands at 14 000–17 000 (Fig. 1, lane 4). When comparable strips of gel were transferred to nitrocellulose and incubated with RCB 2.2 rat calvaria osteoblast-like cells, cell binding was detected to purified human plasma fibronectin (Fig. 1, lane 5), as expected. In the bone protein extract, good cell binding was detectable in proteins co-migrating with fibronectin and the collagen α -chains and, in addition, prominent cell-binding bands were seen at $M_r \sim 30$ 000 and to the multiple bands between $M_r \sim 14$ 000 and 17 000 (Fig. 1, lane 6). Thus, although every protein band detectable by Coomassie Blue on SDS/polyacrylamide-gel electrophoresis was electrophoretically transferred to nitrocellulose, not every band had cell-binding capacity (Fig. 1, lanes 4 and 6, and Fig. 2, lanes 1 and 2). At higher magnifications, individual cells and cell aggregates are easily detectable (Fig. 2, lane 3). Generally, a few cells bind non-specifically to the bovine serum albumin-coated nitrocellulose strips whether or not proteins have been transferred on to the

Fig. 1. SDS/polyacrylamide-gel electrophoresis (5–20% gradient gel) (lanes 1, 2, 3, 4) and RCB-2.2-cell-binding analysis of corresponding lanes on nitrocellulose strips (lanes 5 and 6).

Lane 1, high- M_r and lane 2, low- M_r markers (Bio-Rad; see the Materials and methods section); lane 3, human plasma fibronectin (FN); lane 4, GdmCl-extracted porcine calvaria and long-bone proteins; lanes 5 and 6, cell binding on fibronectin (5) and bone proteins (6).

Fig. 2. Nitrocellulose strip with bone proteins (lane 1) and corresponding RCB-2.2-cell binding on a duplicate nitrocellulose strip (lanes 2 and 3)

Lane 1, lower portion of a nitrocellulose strip with GdmCl-extracted porcine bone proteins electrophoretically transferred from SDS/7.5%-polyacrylamide-gel electrophoresis and stained with Amido Black; lane 2, corresponding nitrocellulose strip with transferred bone proteins incubated with bovine serum albumin, followed by RCB 2.2 cells, then fixed and stained with Amido Black; lane 3, higher magnification of the indicated portion of lane 2. Note that some protein bands do not show cell-binding activity (single arrowhead), whereas other proteins show intense cell-binding capacity (double arrowheads). Also note that, at higher magnification, individual cells and cell aggregates are clearly detectable within the bands; a few cells and aggregates are visible in the background on other portions of the strip (arrows).

strips; such a small, non-specific, cell binding is consistent with that seen in other assays, e.g. cell attachment on to bovine serum albumin-coated Petri dishes. Occasionally a larger 'smudge' of cells is seen in roughly the centre of the strip. Although more vigorous shaking during cell incubations reduced this non-specific cell binding, such vigorous agitation normally inhibited good cell attach-

ment, even to proteins such as fibronectin; therefore intermittent gentle shaking was routinely used. It should also be noted that cell binding was not observed when incubations were done at 4 °C.

By using the same dissociative extraction procedures as used for bone, we also analysed some soft connective tissues for the presence of low- M_r cell-binding proteins

Fig. 3. SDS/polyacrylamide-gel electrophoresis (5-20% gradient gel) (lanes 1, 2, 3, 4) and corresponding RCB 2.2 cell binding on nitrocellulose strips (lanes 5, 6, 7)

Lane 1, mixture of high- and low- M_r markers; lane 2, GdmCl extracts of porcine calvariae and long bones; lane 3, porcine gingiva; lane 4, porcine skin; lane 5, cell binding on bone proteins; lane 6, gingiva; lane 7, skin.

Fig. 4. SDS/polyacrylamide-gel electrophoresis (5–20% gradient gel) (lanes 1, 2, 3, 4) and cell binding on corresponding nitrocellulose strips

Lane 1, mixture of high- and low- M_r proteins; lane 2, GdmCl-extracted porcine calvariae and long-bone proteins; lane 3, GdmCl-extracted RCB 2.2-cell-layer proteins; lane 4, GdmCl-extracted porcine calvariae and long-bone proteins (similar extract to that in lane 2); lanes 5 and 6, RCB 2.2 cell binding on bone proteins (5) and on RCB 2.2-cell-layer proteins (6); lane 7, PLF-cell binding on bone proteins.

similar to those seen in bone. When RCB 2.2 cells were incubated with nitrocellulose strips containing proteins extracted from porcine gingiva (Fig. 3, lane 3) and porcine skin (Fig. 3, lane 4), very prominent cell attachment was observed at $M_r \sim 30000$ and on multiple bands between $M_r \sim 14000$ and 17000 in both soft tissue extracts (Fig. 3, lanes 6 and 7) and in extracts from periodontal ligament (not shown).

Since low- M_r proteins with attachment properties for osteoblast-like cells were present in extracts from both mineralized and soft connective tissues, we also determined whether these same proteins were recognized by cells derived from soft connective tissues. Thus fibroblast-like cells derived from porcine periodontal ligament were incubated with the bone-matrix extracts, and good binding of these fibroblast-like cells was observed on the same proteins (Fig. 4, lane 7). Finally, we wished to determine whether similar low- M_r cell-attachment proteins could be extracted from the ECM made and deposited by cells in culture. For this purpose, we grew RCB 2.2 cells to confluence on plastic tissue-culture dishes and prepared extracts of cell layers by using the same 4 M-GdmCl dissociative extraction procedure. Interestingly, amongst a large number of proteins detectable in such extracts, specific cell attachment was observed in the M_r range of fibronectin, in the low- M_r range of 30000 , and on multiple bands between M_r 14000 and 17000 that co-migrated with the attachment proteins seen in soft- and mineralized-tissue extracts. In addition, several other discrete cell-binding bands were seen between M_r 17000 and 30000 (Fig. 4, lane 6).

DISCUSSION

When either soft (skin, gingiva) or mineralized (bone) connective tissues are extracted with 4 M-GdmCl, a

number of proteins with cell-binding capacity can be identified. In addition to binding on fibronectin and type I collagen, cell attachment is detectable to protein with $M_r \sim 30000$ and on a family of bands with $M_r \sim 14000$ – 17000 . These low- M_r cell-binding molecules can mediate the attachment of a variety of different connective-tissue cells, including osteoblast-like cells derived from bone, fibroblast-like cells derived from periodontal ligament, tumour-derived osteosarcoma cells (results not shown), as well as non-connective-tissue cells, e.g. epithelial cells (not shown). Their presence in both mineralized and soft connective tissues, and their recognition by normal cells derived from both mineralized and soft connective tissues, by tumour-derived osteosarcoma cells and by epithelial cells, suggests that these molecules may be generally recognized and utilized by a wide variety of cell types.

With regard to the presence of the low- M_r cell-binding proteins in both mineralized and soft connective tissues, it is important to note that the tissues were extracted under dissociative extraction conditions with 4 M-GdmCl. The requirement for such extraction conditions strongly suggests that these cell-binding proteins are closely associated with other matrix macromolecules. Although we have used an initial 4 M-GdmCl extract from tissues for most of our cell-binding studies, when the bone tissues were subsequently extracted with 4 M-GdmCl/0.5 M-EDTA (to extract mineral-binding proteins) and the extracted proteins were analysed under similar gel conditions, a series of low- M_r proteins co-migrated with some of the cell-binding proteins extracted with 4 M-GdmCl alone. Thus it is possible that the proteins with cell-binding properties may be widespread in the tissues, and at least some of them may be closely associated with the hydroxyapatite crystals. Further analysis of the precise tissue distribution of the lower- M_r

(14000–17000) proteins, using monoclonal antibodies against these proteins, is required (T. Sugiyama, J. Farsi, S. Wasi, U. Bhargava, J. Sodek & J. Aubin, unpublished work).

Several proteins with cell-binding properties have already been localized in connective tissues. Fibronectin has been described in most connective tissues (note, however, that it is undetectable in certain oral connective tissues; see, e.g., Connor *et al.* [27]) and in at least some epithelial tissues. Vitronectin has also been found in a variety of tissues, and is particularly evident in loose connective tissue, often appearing in fibrillar networks coincident with those staining with fibronectin antibodies [12]. In spite of its similarities to fibronectin (i.e. heparin-binding site, cell-attachment properties, similar tissue distributions), vitronectin is antigenically distinct from fibronectin, although it has been speculated that they might be related through a common ancestral protein [12]. The protein, or family of proteins, that we describe here will also be interesting to investigate with respect to the cell-binding domain, tissue distribution and interaction with other macromolecules, since they represent the smallest naturally occurring proteins yet described with cell-binding activity, and are in the same M_r range as the cell-binding fragment of fibronectin. Whether the M_r -14000–17000 proteins are related to each other or the larger (30000)- M_r cell-binding protein is presently not known and requires further investigation. However, the proteins do not appear to be fragments of fibronectin, since they are not recognized on immunoblots by polyclonal or several monoclonal fibronectin antibodies (J. Sugiyama, J. Farsi, S. Wasi, U. Bhargava, J. Sodek & T. Aubin, unpublished work), nor do fibronectin antibodies block cell binding to these proteins on nitrocellulose strips, although cell binding to fibronectin itself is diminished (results not shown).

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