THE EFFECTS OF EGTA AND TRYPsin ON THE SERUM REQUIREMENTS FOR CELL ATTACHMENT TO COLLAGEN

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SUMMARY

Cells growing on plastic or glass surfaces in vitro may be brought into suspension by proteases (e.g. trypsin) or chelating agents (e.g. EGTA). Trypsin and EGTA remove different quantities and types of molecules from cell surfaces. Previous studies have revealed that when confluent cultures of either BHK or PyBHK cells are brought into suspension by exposure to trypsin, foetal calf serum (or fibronectin) is required for cell attachment to films of denatured type I collagen, but not to 3-dimensional gels of native collagen fibres. In this communication the serum requirements for the attachment of BHK and PyBHK cells to collagen substrata have been examined as a function of (a) the method used to prepare the cell suspension (EGTA or trypsin), and (b) cell density. Data are presented consistent with the view that cell surface-associated fibronectin is able to mediate cell attachment directly to films of denatured collagen.

INTRODUCTION

Grobstein (1953) first proposed that the extracellular matrix plays an important role in the control of cell behaviour during ontogeny. Collagen is a major constituent of the extracellular matrix and there is a growing body of information, in support of Grobstein's hypothesis, indicating that collagen can have a marked effect in vitro on cell differentiation (Konigsberg & Hauschka, 1965; Meier & Hay, 1974; Kosher & Church, 1975), proliferation (Svotelis, Foard & Bang, 1974; Liotta et al. 1978), and migration (Bard & Hay, 1975; Stenn, Mackie & Roll, 1979). Although the means by which collagen exerts these effects on cell behaviour are not understood, it is clear that cell attachment to the collagen substratum is required (Meier & Hay, 1975; Lash & Vasan, 1977; Bunge & Bunge, 1978), and it is for this reason that studies dealing with the mechanism of cell attachment to collagen assume a particular importance.

Klebe (1974) observed that serum was required for the attachment of SV3T3 cells to films of type I collagen (pretreated with 8 M urea) and was able to isolate the glycoprotein from serum responsible for this activity. A similar glycoprotein capable of mediating cell attachment to films of collagen was isolated from the surface of BHK cells (Pearlstein, 1976) and subsequent work in a number of laboratories has demonstrated that the serum-derived and cell surface-derived glycoproteins described by these authors are immunologically indistinguishable and now collectively referred to as fibronectin (Yamada & Olden, 1978; Vaheri & Mosher, 1978; Grinnell 18-2
More recent studies have indicated that serum or fibronectin is actually only required for the attachment of cells to films of denatured type I collagen and not to native collagen fibres (Schor & Court, 1979; Grinnell & Minter, 1978). All of the above studies concerned with the requirements for cell attachment to collagen have employed trypsin in preparing the cell suspension to be used in the attachment assay. Cell suspensions can also be prepared using chelating agents such as EDTA (ethylenediamine tetra-acetic acid) and EGTA (ethyleneglycol-bis-(β-aminooxyethyl ether) N,N’-tetra-acetic acid). Chelating agents remove different amounts and types of surface-associated molecules from cells than trypsin (Snow & Allen, 1970; Codington, Sanford & Jeanloz, 1970; Huet & Herzberg, 1973; Kelley & Lauer, 1975; Anghileri & Dermeitzel, 1976) and cell suspensions prepared by these 2 means display differences in the kinetics of cell attachment to cellular monolayers (Brugmans, Cassiman & Van den Berghe, 1978), cell aggregation (Cassiman & Bernfield, 1974), and cell agglutination by lectins (Burger, 1969). Trypsin may also be internalized by the cell and its action therefore not restricted to the cell surface (Hodges, Livingston & Franks, 1973).

The purpose of this study is to compare the effects of EGTA and trypsin on the attachment of BHK and PyBHK cells to films of denatured collagen and 3-dimensional gels of native collagen fibres.

**MATERIALS AND METHODS**

**Cells and culture conditions**

BHK21/C13 and polyoma virus-transformed cells (PyBHK) were grown in Eagle's minimal essential medium (MEM) supplemented with 10% foetal calf serum, 1 mM sodium pyruvate, 2 mM glutamine, non-essential amino acids (Gibco Bio Cult, Ltd., Glasgow, cat. no. 114) and 100 units/ml penicillin and streptomycin. Stock cultures growing in 90-mm plastic Petri dishes (Gibco Bio Cult, Ltd., Uxbridge, cat. no. 1415) were subcultured once a week and medium changed 3 times a week. Confluent cultures contained 4-6 x 10⁶ cells/dish and subconfluent cultures 4-6 x 10⁵ cells/dish at harvesting.

**Preparation of collagen substrata**

An aqueous solution of type I tropocollagen was prepared from rat tail tendons as described by Elsdale & Bard (1972). The optical density of the tropocollagen solution was measured at 230 nm and the concentration of tropocollagen calculated using a calibration curve prepared from standard solutions of freeze-dried rat tail tendon collagen extracted in the same manner. The concentration of tropocollagen in the aqueous stock solutions was adjusted to between 2.0 and 2.5 mg/ml prior to use.

Three-dimensional gels of native collagen fibres were prepared in 35-mm plastic tissue culture Petri dishes (Gibco Bio Cult, Ltd., Uxbridge, cat. no. 53666), from the aqueous tropocollagen stock solution according to Elsdale & Bard (1972). Heat-denatured collagen was prepared by incubating the tropocollagen solution at 60 °C for 30 min and quickly lowering the temperature by immersion into an ice bath. Films of heat-denatured collagen were made by covering the bottoms of 35-mm plastic tissue culture Petri dishes with the solution of heat-denatured collagen, removing excess liquid with a Pasteur pipette and incubating the dishes for 20 min in a dessicator containing an open beaker of concentrated ammonia. The films were then air-dried and washed 5 times with distilled water before use. The arrangement of collagen molecules within these films is discussed in Schor & Court (1979).
Preparation of single cell suspensions and determinations of the kinetics of cell attachment

Separate stock solutions of 2 mM EGTA and 0.25% crystalline trypsin were prepared in Dulbecco’s phosphate-buffered saline (PBS). Cell cultures were washed 3 times with Hanks’ balanced salt solution and then incubated at 37 °C with either (a) 4 ml of 2 mM EGTA for 20 min, or (b) 4 ml of 2 mM EGTA for 20 min, followed by the addition of 1 ml of 0.25% trypsin (giving a final concentration of 0.05% trypsin) and continuing the incubation for an additional 3 min. One milliliter of 0.25% soybean trypsin inhibitor was then added to all dishes, the cells pipetted several times to produce a uniform single cell suspension, centrifuged at 300 g for 5 min, resuspended in serum-free MEM and the concentration adjusted to approximately $1 \times 10^4$ cells/ml. Untreated plastic tissue culture dishes and dishes containing the collagen substrata were incubated for 1 h at 37 °C with 1 ml of either serum-free MEM or MEM containing 10% foetal calf serum. At the beginning of the experiment 1 ml of cell suspension was added to these dishes, thus giving a cell suspension in either serum-free MEM or MEM containing 5% foetal calf serum. The dishes were kept at 37 °C in a humidified CO$_2$ incubator and the number of unattached cells determined at the beginning of the experiment (i.e., total number of cells/dish) and at various times thereafter by transferring the medium (2 ml) from each dish to a vial containing 5 ml of 'Isoton' (Coulter Electronics, Ltd., Harpenden, Herts.), gently washing the dishes 3 times with Hanks’ balanced salt solution (3 ml in total), adding these washes to the vial and finally determining the number of cells recovered in this manner with a Coulter electronic particle counter. The number of attached cells at any time could then be calculated by subtracting the number of unattached cells from the total cell number per dish. Data are expressed as the percentage of cells attached. The kinetics of cell attachment must be calculated in this manner since cells attached to native collagen fibres are not completely removed by exposure to trypsin (Schor & Court, 1979).

Chemicals

Crystalline trypsin (T-8253), soybean trypsin inhibitor (T-9023) and EGTA (E-4378) were all obtained from Sigma Chemical Co., London.

RESULTS

BHK and PyBHK cells brought into suspension by trypsin have been reported to require the presence of serum (or fibronectin) for attachment to films of denatured type I collagen, but not to native collagen fibres (Schor & Court, 1979; Grinnell & Minter, 1978). In the experiments presented here, confluent cultures of BHK and PyBHK cells were brought into suspension by exposure either to EGTA only or EGTA and trypsin, and then used to measure the kinetics of cell attachment to collagen substrata in the presence of serum-free growth medium or growth medium containing 5% foetal calf serum. The results obtained in a representative experiment with confluent cultures of PyBHK cells are shown in Fig. 1. The kinetics of attachment to the different substrata of PyBHK cells treated with EGTA only are indistinguishable from those obtained with cells treated with trypsin. Different results are obtained with confluent cultures of BHK cells (Fig. 2). In this case cells treated with EGTA only no longer require serum for attachment to films of denatured collagen.

The results obtained in 5 such experiments are presented in Table 1. Data are expressed as (a) the mean ± S.D. of the percentage of cells attached to the various substrata after 120 min of incubation, and (b) the difference between the percentage attached to each substratum in the presence and absence of serum. These results
Fig. 1. The attachment of PyBHK cells from confluent cultures to collagen substrata. PyBHK cells were harvested from confluent cultures by exposure either to EGTA (A–C) only or EGTA + trypsin (D–F), as described in Materials and methods. These cells were then used to measure the kinetics of cell attachment to plastic tissue culture dishes (A, D), films of denatured type I collagen (B, E) and 3-dimensional gels of native collagen fibres (C, F) either in serum-free MEM (○) or MEM containing 5 % foetal calf serum (●). Data are expressed as the percentage of cells attached after 30, 60 and 120 min of incubation.

Fig. 2. The attachment of BHK cells from confluent cultures to collagen substrata. BHK cells were harvested from confluent cultures by exposure either to EGTA (A–C) only or EGTA + trypsin (D–F), as described in Materials and methods. These cells were then used to measure the kinetics of cell attachment to plastic tissue culture dishes (A, D), films of denatured type I collagen (B, E) and 3-dimensional gels of native collagen fibres (C, F) either in serum-free MEM (○) or MEM containing 5 % foetal calf serum (●). Data are expressed as the percentage of cells attached after 30, 60 and 120 min of incubation.
indicate that the attachment of confluent BHK cells treated with EGTA only to films of denatured collagen is not serum-dependent, whereas the attachment of cells removed by EGTA and trypsin shows a significant serum-dependence, comparable to that observed with PyBHK cells treated with either EGTA or EGTA and trypsin. The attachment of BHK and PyBHK cells to gels of native collagen fibres is not dependent on serum under any of the conditions tested.

Table 1. The attachment of PyBHK and BHK cells from confluent cultures to collagen substrata

<table>
<thead>
<tr>
<th>Cell</th>
<th>Substratum</th>
<th>Serum</th>
<th>% attachment</th>
<th>Δ attached (+ serum)</th>
<th>Δ attached (- serum)</th>
<th>EGTA</th>
<th>EGTA + trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PyBKH</td>
<td>Plastic</td>
<td>+</td>
<td>84.7 ± 0.6</td>
<td>-6.7 ± 1.5</td>
<td>13.5 ± 5.8</td>
<td>77.7 ± 6.9</td>
<td>75.6 ± 11.1</td>
</tr>
<tr>
<td></td>
<td>Denatured</td>
<td>-</td>
<td>91.4 ± 2.1</td>
<td></td>
<td></td>
<td>91.2 ± 4.5</td>
<td>14.0 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>collagen films</td>
<td>+</td>
<td>83.3 ± 4.8</td>
<td>47.3 ± 6.2</td>
<td>35.2 ± 11.5</td>
<td>75.6 ± 11.1</td>
<td>14.0 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>Native collagen</td>
<td>-</td>
<td>83.9 ± 9.7</td>
<td>-0.9 ± 3.2</td>
<td>-2.5 ± 3.0</td>
<td>73.4 ± 3.0</td>
<td>17.5 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>gels</td>
<td>-</td>
<td>84.8 ± 6.5</td>
<td></td>
<td></td>
<td>75.9 ± 5.1</td>
<td>17.5 ± 5.1</td>
</tr>
<tr>
<td>BHK</td>
<td>Plastic</td>
<td>+</td>
<td>82.2 ± 0.0</td>
<td>-5.7 ± 7.5</td>
<td>-9.1 ± 4.8</td>
<td>82.3 ± 7.1</td>
<td>91.4 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>Denatured</td>
<td>-</td>
<td>87.9 ± 6.1</td>
<td></td>
<td></td>
<td>91.4 ± 5.7</td>
<td>14.0 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>collagen films</td>
<td>+</td>
<td>81.3 ± 8.0</td>
<td>8.6 ± 10.7</td>
<td>38.4 ± 11.9</td>
<td>75.6 ± 8.4</td>
<td>13.7 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>Native collagen</td>
<td>-</td>
<td>72.7 ± 10.5</td>
<td>15.4 ± 8.3</td>
<td>0.2 ± 8.0</td>
<td>84.8 ± 4.3</td>
<td>85.0 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>gels</td>
<td>-</td>
<td>83.3 ± 3.8</td>
<td></td>
<td></td>
<td>85.0 ± 3.9</td>
<td>13.7 ± 11.3</td>
</tr>
</tbody>
</table>

PyBHK and BHK cells were harvested from confluent cultures by exposure either to EGTA or EGTA + trypsin, as described in Materials and methods. These cells were then used to measure the kinetics of cell attachment to plastic tissue culture dishes, films of denatured collagen and 3-dimensional gels of native collagen fibres either in serum-free MEM or MEM containing 5% fetal calf serum. Data are presented as (a) the mean ± s.d. of the percentage of cells attached after 120 min of incubation, and (b) the difference between the percentage of cells attached to each substratum in the absence of serum from that observed in the presence of serum (a negative number therefore indicates that more cells attached in the absence of serum than in the presence of serum). Data from 5 experiments are summarized in this table.

The results obtained in 3 experiments with subconfluent BHK cells are shown in Table 2. In contrast to the results obtained with confluent cultures, subconfluent BHK cells treated with either EGTA only or EGTA and trypsin show a significant serum-dependence of attachment to films of denatured collagen. The attachment of subconfluent BHK cells to gels of native collagen fibres is again not dependent on serum. The results obtained with subconfluent cultures of PyBHK cells (data not shown) are basically the same as shown in Table 1 for confluent cells.
Table 2. The attachment of BHK cells from subconfluent cultures to collagen substrata

<table>
<thead>
<tr>
<th>Substratum</th>
<th>Serum</th>
<th>% attachment</th>
<th>Δ attached (serum)</th>
<th>Δ attached (-serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic</td>
<td>+</td>
<td>79 ± 1.3</td>
<td>86 ± 2</td>
<td>191 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>89 ± 1.3</td>
<td>96 ± 2.2</td>
<td>97 ± 0.9</td>
</tr>
<tr>
<td>Denatured collagen films</td>
<td>+</td>
<td>73 ± 1.1</td>
<td>78 ± 3.7</td>
<td>73 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>37 ± 3.1</td>
<td>40 ± 1.8</td>
<td>37 ± 2.0</td>
</tr>
<tr>
<td>Native collagen gel</td>
<td>+</td>
<td>86 ± 2.0</td>
<td>81 ± 0.9</td>
<td>87 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>86 ± 1.1</td>
<td>84 ± 3.0</td>
<td>84 ± 1.0</td>
</tr>
</tbody>
</table>

BHK cells were harvested from subconfluent cultures by exposure either to EGTA or EGTA + trypsin. These cells were then used to measure the kinetics of cell attachment to plastic tissue culture dishes, films of denatured type I collagen and 3-dimensional gels of native collagen fibres either in serum-free MEM or MEM containing 5% foetal calf serum.

DISCUSSION

Fibronectin is a major surface constituent of normal fibroblasts, but is absent from the surface of their virally transformed counterparts (Pearlstein & Waterfield, 1974; Hynes & Humphreys, 1974; Gahmberg, Kiehn & Hakomori, 1974; Hynes, 1976; Vaheri & Mosher, 1978; Yamada & Olden, 1978). The morphological distribution and quantity of fibronectin on cell surfaces varies as a function of cell density in culture, with more fibronectin being associated with confluent fibroblasts than with subconfluent ones (Pearlstein & Waterfield, 1974; Mautner & Hynes, 1977; Furcht, Mosher & Wendelschafer-Crabb, 1978).

Fibronectin is also found in serum or plasma, where it is referred to as cold insoluble globulin or CIG (Grinnell & Hays, 1978). Although the cell surface-derived and serum-derived forms of fibronectin are immunologically indistinguishable and are equally effective in mediating cell attachment to films of denatured collagen (Yamada & Kennedy, 1979), important differences between these two forms of fibronectin have been observed; these include differences in solubility, electrophoretic mobility and ability to agglutinate red blood cells (Yamada & Kennedy, 1979; Yamada & Olden, 1978). Perhaps the most relevant difference between the cell surface-derived and serum-derived forms of fibronectin in the context of the present discussion is the reported inability of serum-derived fibronectin to bind directly to cell surfaces (Pearlstein, 1978), whereas the cell surface-derived form will bind to cells and when added to cultures of transformed fibroblasts results in a restoration of some of the growth characteristics of normal cells (Yamada, Yamada & Paston, 1976). A considerable amount is known about the means by which serum-derived fibronectin mediates cell attachment to films of denatured collagen. This form of fibronectin binds avidly to collagen at a site between residues 757 and 791 on the α1(I) chain.
Cell attachment to collagen

(Kleinman et al. 1978) and significantly more fibronectin binds to denatured collagen than to native collagen fibres (Engvall & Rouslahti, 1977). As mentioned above, serum-derived fibronectin does not bind directly to cell surfaces, and mediates cell attachment to denatured collagen by first forming a complex with the collagen substratum, followed by cell attachment to this fibronectin-collagen complex (Klebe, 1974). Pearlstein (1978) has suggested that the interaction of fibronectin with collagen induces a conformational change in the fibronectin molecule required for recognition by receptors on the cell surface.

Cell surface fibronectin is extremely sensitive to trypsin and exposure of confluent cultures of BHK cells to 10 μg/ml trypsin (50-fold less than used in this study) has been observed to remove all detectable surface-associated fibronectin (Pearlstein & Waterfield, 1974). On the other hand, cell surface fibronectin is not sensitive to chelating agents such as EDTA and EGTA (Pearlstein & Waterfield, 1974). The results reported in this communication indicating that confluent BHK cells removed by exposure to EGTA only do not require serum for attachment to films of denatured collagen, while such cells removed by EGTA and trypsin do require serum, are consistent with the view that cell surface-associated fibronectin can mediate cell attachment directly to denatured collagen. PyBHK cells and subconfluent BHK cells either do not have surface-associated fibronectin or have an insufficient amount of it and therefore require exogenous fibronectin to mediate cell attachment to denatured collagen, regardless of whether or not trypsin is used to prepare the cell suspension. In contrast, cell attachment to gels of native collagen fibres occurs by a fibronectin-independent mechanism (Schor & Court, 1979). This conclusion is supported by the results presented here indicating that there are no differences in the kinetics of attachment of both BHK and PyBHK cells following treatment with either EGTA only or EGTA and trypsin to native fibres. These results also indicate that although EGTA and trypsin remove different components of the cell surface, including proteins and proteoglycans, none of these appear to influence the ability of cells to attach to native fibres in the presence or absence of serum.

REFERENCES


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