Changes in the surface coat of mesenchymal cells of mouse limb buds after enzymatic cell separation

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SUMMARY

Isolation of cells is nowadays performed by enzymatic means. The influence of such enzymes on the surface coat of mesenchymal and blastemal cells during the dissociation of limb buds from 11-day-old mouse embryos was studied electron microscopically after staining with ruthenium red. EGTA or collagenase failed to bring about cell separation. The surface coat seemed to be unchanged after collagenase treatment. After EGTA an increase in extracellular filaments was observed. The proteases α-chymotrypsin, dispase II, papain, pronase P and trypsin (0.2%, 37 °C, 20 min) succeeded in completely dissociating limb buds. Apart from single granules, there was a detachment of the surface coat from the cells in all cases studied. Hyaluronidase led to only partial separation, but the detachment of the surface coat was almost complete, indicating a GAG-rich surface layer on these cells.

INTRODUCTION

Enzymatic dissociation of an organ into single cells is a common means for the preparation of primary cell cultures from solid organs (Moscona, 1952; Lasfargues, 1956; Rinaldini, 1958). In most cases proteases such as trypsin or pronases, or specific enzymes such as elastase (Rinaldini, 1959) or collagenase and hyaluronidase (Seglen, 1972) are used. Ca²⁺ deficiency with EDTA or EGTA may also lead to organ dissociation. In a number of cases a chelating agent is combined with a protease, or a Ca²⁺- and Mg²⁺-free medium is used (Moscona, 1952; Wiepjes & Prop, 1970).

During organ isolations not only disintegration of the intercellular matrix but also detachment of the cellular contacts (Fukuyama, Black & Epstein, 1974) and formation of microvilli on the cells (Follet & Goldman, 1970) are observed. In addition, detachment of glycosaminoglycans (GAG) and glycoproteins from the surface coat of the cells takes place (Kraemer & Tobey, 1972; Ceriani, Petersen & Abraham 1978). Concomitantly the electrophoretic motility of freshly isolated cells changes (Barnard, Weiss & Ratliffe, 1969; Maslow, 1970) as well as cell adhesion, cellular growth (Poste, 1971) and the reaggregation behaviour of the cells (Wiseman & Hammond, 1976).

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The surface coat, consisting of oligosaccharide chains of membrane-integrated glycoproteins, of glycolipids, and of membrane-associated proteoglycans and GAGs (Rambourg, 1971; Bretscher, 1973; Sturgess, Moscarello & Schachter, 1978), can be observed by different methods (e.g. ruthenium red) and has been seen in all cells so far studied (Rambourg, Neutra & Leblond, 1966; Rambourg & Leblond, 1967; Luft, 1966; Behnke, 1968; Friess & Liebich, 1972; Latta, Johnston & Stanley, 1975).

Numerous functions are attributed to this cell surface coat during cell recognition, adhesion and migration as well as in the communication between cells and extracellular spaces (cf. Marchesi, Ginsburg, Robbins & Fox, 1978; McClain & Edelman, 1978).

The surface coat is of great significance in the behaviour of the cells during morphogenesis and in vitro. Therefore we investigated the surface coat of the cells after isolation by electron microscopy. We performed these studies on embryonic mesenchymal and blastemal cells of mouse limb buds. In this tissue the surface coat is expected to be of great morphogenetic significance. Furthermore, the morphology of this layer is not as yet exactly understood.

MATERIALS AND METHODS

NMRI mice were kept at a reversed day/night cycle. The day of conception was considered as day 0 of development. The upper limb buds of 11-day-old mouse embryos were cut off at the trunk, rinsed once in Hanks BSS and subsequently incubated for 20 min in 20 ml enzyme solution at 37 °C in a water bath at a shake frequency of 90/min. For cell isolation the suspension was pipetted four to five times, made up with Hanks BSS to double its original volume and centrifuged at 50 x g for 10 min. The cell sediment was washed once in Hanks BSS and then fixed in ruthenium red/glutaraldehyde. Ruthenium red was used for demonstration of the surface coat, because it leads to intensification of contrast of glycosaminoglycans and proteoglycans in the electron microscope (Luft, 1966; Rambourg, 1971; Kelley & Lauer, 1975).

Fixation was done according to Luft (1972): 1 h in 1% glutaraldehyde with 1.5 g ruthenium red/l in 0.1 M cacodylate buffer pH 7.4, rinsing in 0.1 M cacodylate buffer with 800 mg ruthenium red/l, postfixation for 1 h in 1% OsO4 with 400 mg ruthenium red/l in 0.1 M cacodylate buffer. This was followed by thoroughly rinsing in 0.9% NaCl, dehydration in acetone and embedding in Mikropal (Ferak, Berlin, Germany). Thin sections were prepared on an LKB-microtome; post-staining was performed with lead citrate and uranyl acetate in aqueous solution. Pictures were made using a Siemens 101 and a Zeiss EM9 electron microscope.
The following solutions were used

Ca- and Mg-free solution (CMF: 8.0 g/l NaCl, 0.3 g/l KCl, 0.05 g/l NaHPO₄, 0.25 g/l KH₂PO₄, 1.0 g/l NaHCO₃, 2.0 g/l glucose (Wiepjes and Prop, 1970). 2 mM EGTA (Serva, Heidelberg, Germany) in CMF; 0.2% α-chymotrypsin, 45 U/mg (Serva, Heidelberg, Germany) in CMF; 0.2% collagenase, 0.15 U/mg (Boehringer, Mannheim, Germany) in Hanks BSS; 0.2% dispase II, 0.5 U/mg (Boehringer, Mannheim, Germany); 0.2% hyaluronidase, 250 USP-U/mg (Merck, Darmstadt, Germany) in CMF; 0.2% papain, 3.5 m Anson-U/mg (Merck, Darmstadt, Germany) in CMF; 0.2% pronase P, 45000 PUK-U/mg (Serva, Heidelberg, Germany) in CMF; 0.2% trypsin 1:250, 4 U/mg (Serva, Heidelberg, Germany) in CMF.

RESULTS

Treatment of 11-day-old mouse limb buds with Ca²⁺- and Mg²⁺-free solution (CMF) failed to bring about cell separation. The electron microscopical picture corresponded to that of untreated controls: in the peripheral and distal regions of the limb buds loosely packed, undifferentiated mesenchymal cells with irregular nuclei, organelle-poor, ribosome-rich cytoplasm and irregular cell processes were observed (Fig. 1a).

At higher magnification a surface coat could be recognized on the cell membrane of these cells. This surface coat either formed a regular layer or consisted of isolated granules or plaques (Fig. 1b). Indentations, augmentation and intensification of staining was often observed on the membrane.

At high magnification a surface coat of about 25 nm thickness became recognizable. It clearly shows a filamentous structure linked to the membranous bilayer. More concentrated areas of surface coat alternated with less concentrated ones (Fig. 1c).

In the central regions of the limb buds the formation of the blastema was already completed at this stage. The cells were densely packed; many ribosomes were still to be seen in the cytoplasm, and mitochondria and rough endoplasmic reticulum occurred to an ever increasing extent (Fig. 2a).

At higher magnification single granules of ruthenium-red-stainable material were visible at membranes in the intercellular space. Between membranes of adjacent cells a thin layer of surface coat was recognizable (Fig. 2b). After treatment with 2 ml EGTA in CMF cell separation was not successful. But in contrast to controls, electron-dense, about 5 nm-thick filaments were seen, which extended far into the extracellular space and inserted into adjacent cells (Fig. 3).

Treatment with various proteases (α-chymotrypsin, dispase II, papain, pronase P, trypsin) in all cases led to complete isolation. The electron microscopical picture of the cell membrane was uniform. There was an almost
complete loss of the surface coat. But single 25 nm granules with fine radiating filaments were still present (Fig. 4). Lesions of the membranous bilayer were never detected.

After trypsin treatment one peculiarity was striking. While, after the other proteases part of the surface coat material was distributed in small fragments almost evenly in the extracellular space, after trypsin an almost complete detachment of the surface coat over wide areas seemed to have taken place. Under these conditions, a continuous surface coat layer, which was detached from the cell membrane over long distances, was often seen (Fig. 5). After an 0.1% trypsin concentration this effect was especially pronounced.

After hyaluronidase, dissociation of the blastema was incomplete. Even after thorough pipetting tissue fragments remained. The single cells that were obtained after sedimentation of these fragments from the supernatant showed on the cell membrane clearly visible remainders of the ruthenium-red-positive surface coat, which were thinner (15 nm) than those on untreated cells (Fig. 6).

After collagenase treatment dissociation of the blastema was not observed. There were units of mesenchymal and blastemal cells as in controls. Their surface coat was unchanged.

**DISCUSSION**

An electron-dense, about 25 nm-thick, surface coat is seen on the cell membrane of blastemal and mesenchymal cells of 11-day-old mouse embryos after ruthenium-red staining. It seems certain, that this ruthenium-red stainable coat is strongly correlated to the biochemical findings on the membrane glycoproteins and GAGs. This surface coat either consists of a continuous layer, or granules and plaques. According to what is known of the structure of the surface coat, sensitivity towards proteolytic treatment is to be expected (Bretscher, 1973; Sturgess et al. 1978).

In our experiments, too, this layer disappears after cell isolation with the different proteases. However, attribution of the isolated cells to the mesenchymal or blastemal part is no longer possible. In all cells investigated the same

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**Figure 1**

(a) Mesenchymal cells of limb buds from 11-day-old mouse embryos. The irregularly shaped cells have an oval, sometimes lobular, nucleus, homogenous cytoplasm and coarse cell processes. N = nucleus, C = cytoplasm, M = unstructured matrix. × 3600.

(b) At higher magnification after ruthenium red staining, an irregular, partly continuous (arrow heads), partly granular (arrows) surface coat is observed, which is concentrated in cell indentations (dark arrows). N = nucleus, C = cytoplasm. × 9200.

(c) At high magnification the filamentous structure of the surface coat can be demonstrated (arrows). Besides loosely packed areas, aggregations of the surface coat (arrow heads) are seen. The average thickness is about 25 nm. × 150000.
Fig. 2(a). Blastema of the limb bud from an 11-day-old mouse embryo. Cells with round-oval nuclei (N) in close contact, only little extracellular space (arrow heads): × 3600. (b) At higher magnification surface coat granules are detected at the membrane with empty extracellular space (arrows) and in a uniform layer between the cells (arrowheads): N = nucleus, C = cytoplasm, M = mitochondria (× 19000).
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Effect is seen: proteases cause complete dissociation of limb buds. Except for a few granules, the ruthenium-red-positive surface coat disappears. We do not assume that cell isolation is only possible when the whole surface coat is destroyed by proteolytic treatment. Detachment of the surface coat should rather be considered as an additional effect of the proteases, since trypsin treatment at +4 °C also causes dissociation of the different tissues without, however, reducing the surface coat which can be demonstrated with ruthenium red (Caravita & Zacchei, 1974). This is also supported by the observation that with trypsin treatment at low temperatures (+4 °C, +15 °C) number and size of colonies are increased after culture of isolated cells (McKeehan, 1977). It is possible that under these conditions only substances which are responsible for adhesion are disintegrated. Investigations on other cell types support this assumption. Although extensive detachment of the surface coat can be achieved with trypsin (Onodera & Sheinin, 1970; Anghileri & Dermietzel, 1976; Cox Bour & Haenelt, 1977; Vogel, 1978) or papain (Forstner, 1971), in certain systems complete detachment of the surface coat cannot be reached. Some glycoproteins on the membrane of erythrocytes (Jackson, Seegrest & Marchesi, 1971) as well as on the membrane of fibroblasts in vitro (Huet & Herzberg, 1973; Kelley & Lauer, 1975) are resistant towards trypsin treatment. These differences may be attributed to a different concentration or activity of trypsin (Huggins, Chestnut, Durham & Caraway, 1976; Friedlander & Fischman, 1977) and to a different composition of glycoproteins in the surface coat of various cell types.

In the cells investigated in the present studies, surface structures which do not detach after proteases and therefore possibly represent glycolipids could be demonstrated in all cases.

The resistance of the surface coat towards chelating agents has been described by many authors (Huet & Herzberg, 1973; Kelley & Lauer, 1975; Cox et al., 1977; Vogel, 1978). It can therefore be assumed that the components of the surface coat are not linked via ionic linkages but covalently bound (Vogel & Dolde, 1979). The formation of fine filaments described here after EGTA indicates that Ca2+ may have a function during inner organization and stabilization of the surface coat. This is also supported by the fact that about 90 % of the total Ca2+ of HeLa cells are localized in the cell coat (Borle, 1968).

Hyaluronidase caused detachment of a large amount of the surface coat without yielding satisfactory cell isolation. Cell adhesion in the limb bud blastema therefore does not primarily depend upon the effect of GAGs, but a large proportion of the surface coat seems to consist of GAGs. In other cells, too, above all in monolayer cells in vitro, GAGs were observed in the surface coat (Vogel & Kelley, 1977; Kraemer & Barnhart, 1978, Barnhart, Cox & Kraemer, 1979). The high percentage of hyaluronidase-soluble material in the surface coat of limb bud cells seems to be interesting in view of the findings of Toole and co-workers (Toole, 1972; Toole, Jackson & Gross 1972) indicating that hyaluronate stimulates cell migration in the mesenchyme and inhibits
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Fig. 7. An untreated mesenchymal cell shows a continuous layer of ruthenium red-stainable material of about 20 nm thickness. $\times 56000$.

Figures 3–6

Fig. 3. Mesenchymal cells of limb buds treated with 2 mM EGTA for 20 min at 37 °C. The surface coat seems to be thicker and more loosely packed. Fine filaments are clearly seen. These protrude into the extracellular space and insert into adjacent cells (arrows). $C =$ cytoplasm, $M =$ unstructured matrix. $\times 56000$.

Fig. 4. Treatment with 0.2% α-chymotrypsin led to complete dissociation into single cells. Except for a few granules (arrows) complete detachment of the surface coat is seen. Similar results are obtained with papain, pronase P, dispase II and trypsin. $C =$ cytoplasm with free ribosomes. $\times 56000$.

Fig. 5. This picture shows partial detachment (arrow) of a continuous layer (arrow heads) of surface coat material after treatment with 0.2% trypsin. $C =$ cytoplasm. $\times 19000$.

Fig. 6. Treatment with 0.2% hyaluronidase does not cause complete detachment of the surface coat. Small amounts of the surface coat material are still recognizable on the cell membrane (arrow heads). $C =$ cytoplasm. $\times 56000$. 
differentiation. Consequently, a high concentration of hyaluronic acid may be expected in the surface coat of undifferentiated cells. Collagen, on the other hand, does not play an essential role for these cells; collagenase therefore does not have any effect on tissue dissociation or on the surface coat.

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