Serum-dependent avian skin differentiation in vitro: time sequence of induced events

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SUMMARY

Information concerning the developmental mechanisms involved in skin differentiation have been mainly derived from in vitro experiments. We have previously observed that 6-day chick embryonic thigh skin keratinizes in vitro in chicken serum-containing medium, but does not do so in chick embryo extract-containing medium. This system seems adequate in many respects for investigating the mutual regulative relationship between epithelium and mesenchyme (so called epithelio-mesenchymal interactions).

We have therefore attempted better to define the time course of differentiative events and their dependence upon the time of serum administration.

Skin explants have been sequentially supplemented with differentiation-stimulating or non-stimulating nutrient, removed at different intervals and examined by histological and histochemical procedures.

Different epithelial and mesenchymal behaviour results according to serum supplementation time. Serum administered in the first 48 h of in vitro maintenance is unable to stimulate either the subsequent epidermal keratinization or the correlated changes in dermal histochemical pattern. On the other hand, serum-containing medium induces keratinization and changes in dermal intercellular composition (glycoproteins accumulate to a greater extent than glycosaminoglycans, as does hyaluronic acid relative to chondroitin sulphuric acids), if added for only the second two days. In the last 48 h, serum may promote epidermal keratinization provided that in vitro incubation has been prolonged.

Administration of actinomycin D with serum in the second 48 h prevents epidermal keratinization and modifies dermal histochemical reactivity, but is ineffective if added in the last 48 h.

The above findings demonstrate that serum factor(s) stimulating epidermal differentiation act(s) on skin explants in the central incubation period and that correlatively mesenchyme acquires a characteristic histochemical pattern, supporting the possibility that the effect of serum may be mediated by changes in the composition of dermal ground substance.

INTRODUCTION

Avian skin has been used extensively in a variety of studies concerned with epidermal differentiation. By means of in vitro experiments, the dependence of chick epidermal differentiation upon the underlying mesenchyme has been well established (McLoughlin, 1961a; Wessels, 1963; Fitton Jackson & Fell, 1963;

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Dodson, 1967; Jensen & Mottet, 1970). These investigations also showed relevant differences in differentiation of skin explants according to embryonic age, body region and culture conditions (see Carinci, Simonelli, Bubola & Pettazzoni, 1976, for a discussion), thus indicating that availability of a well defined system is a prerequisite to the study of the regulatory role of mesenchyme.

In previous work we have established such a system comprising 6-day chick embryo thigh skin maintained in vitro in different nutrients. 6-day thigh skin explants supplemented with serum-containing nutrient undergo keratinization faster than in vivo. As judged by histological, histochemical and ultrastructural criteria, this keratinization is very similar to that in vivo. On the other hand, epidermal differentiation does not take place when skin explants are maintained in chick embryo extract-containing medium (Carinci & Simonelli, 1972; Carinci et al. 1976). In addition, in the differentiating explants dermal ground substance changes its histochemical and biochemical pattern correlative with the keratinization (Carinci, Pane, Simonelli & Zaniboni, 1975) and in a manner similar to that observed during in ovo keratinization (Pane, Becchetti & Carinci, 1974; Kawamoto & Nagai, 1976). Finally, ultrastructural data suggest that epidermal differentiation in vitro mainly comprises a two-step process, i.e. ‘basal’ and ‘specific’ steps, only the second being under the mesenchymal control (Carinci et al. 1976).

Two main conclusions may be drawn from the above findings: the dependence of both epithelial and mesenchymal differentiation on environmental factors, and an actual correlation between the onset of epidermal keratinization and biochemical and histochemical changes in the underlying mesenchyme.

In the current research, we have tried to obtain additional information on the time sequence of in vitro inductive events, taking advantage of different ability of the two nutrients to promote epithelial keratinization. We have therefore carried out experiments sequentially supplying skin explants with either medium, in order to evaluate the dependence of epidermal keratinization and dermal histochemical composition upon the time of serum addition.

Recently, it has been demonstrated that serum controls mucopolysaccharide production by stationary chick fibroblasts in culture and that actinomycin D prevents this effect (Kurtz & Stidworthy, 1975). Since in previous work it has been observed that actinomycin D was able to inhibit epidermal differentiation (Zambonelli-Simonelli, Caruso, Mosca Bubola & Pettazzoni, 1972), we have also investigated whether the effect of this antimetabolite could be related to a change in dermal intercellular composition.

The present results enable us better to define the timing of the sequential events involved in epidermal differentiation in vitro and to further substantiate the suggestion of a possible regulatory role of mesenchymal intercellular components (Carinci et al. 1976).
MATERIAL AND METHODS

White Leghorn fertilized eggs, provided by the Corticella Agricultural Station (Bologna), were incubated at 38 °C and 60 % relative humidity. Skin areas, about 1.5 x 1.5 mm², were carefully dissected under sterile conditions from the thigh regions of 6-day embryos, staged according to the Hamburger-Hamilton table (Hamilton, 1952), and then placed in culture dishes on the vitelline membrane according to the technique of Wolff (1961).

Nutrients were as follows: (a) chicken serum nutritional medium (9 drops gelose 1 % in Gey fluid, 3 drops chicken serum (Difco); 1 drop penicillin and streptomycin solution, 100 units/ml, Gibco), thereafter referred to as CS; (b) chick embryo extract nutritional medium (9 drops gelose 1 % in Gey fluid, 3 drops chick embryo extract (E 100, Difco), 1 drop penicillin and streptomycin solution, 100 units/ml, Gibco), thereafter referred to as E.

The cultures were incubated at 37 °C. Every second day skin explants were removed together with the supporting vitelline membrane, carefully rinsed in Tyrode's and transferred to another culture dish containing the appropriate nutrient according to the experimental schedule (see Table 1).

Actinomycin D (Sigma Chemical Co.) was incorporated into the nutrients to give 0.05 µg/culture, a concentration previously proven to prevent keratinization without inducing morphological epithelial damage (Zambonelli-Simonelli et al. 1972).

Cultures were fixed in Bouin’s fluid and routine histological procedures were followed. Serial sections were cut at 5–7 µm and stained for morphological examination with hematoxylin-eosin. For the histochemical analysis, we have used a set of procedures in order to detect glycoproteins (GP) and glycosaminoglycans (GAG) and distinguish between different GAGs: periodic acid Schiff (PAS) reaction before and after diastase (Hoechst, 0.1 % in 0.2 M-phosphate buffer, pH 6, 37 °C, 1 h) and dinedone (5 % in absolute ethanol, 5 h, 60 °C) treatments; sequential staining with Alcian blue 8 GX(Fluka) 1 % in 0.3 M or 0.025 M-MgCl₂ solution and PAS; sequential staining with Alcian blue 1 % in 0.3 M-MgCl₂ solution and Alcian yellow GXS (Chroma), 1 % in 0.025 M-MgCl₂ solution. The GAGs were resolved by Alcian staining into different classes owing to the different critical electrolyte concentration at which the anionic polymers change from binding dye to binding Mg²⁺ (Scott & Dorling, 1965). Sections were also incubated with testicular hyaluronidase (Jalovis Vister, 1 mg/ml in 0.1 M-phosphate buffer, pH 7, 6 h, 37 °C). Control sections were incubated in buffer alone.

We assumed that: PAS positively abolished by dinedone treatment and no by diastase digestion was indicative of GP presence; staining with Alcian blue in 0.3 M-MgCl₂ which was not removed by testicular hyaluronidase was indicative of chondroitin sulphate B (dermatan sulphate); staining with Alcian blue in 0.3 M-MgCl₂ which was hyaluronidase sensitive was indicative of chondroitin
sulphates A/C (CSA A/G); staining with Alcian yellow in 0.025 M-MgCl₂ was indicative of hyaluronic acid (HA), the reactivity of more acidic groups being blocked by previous 0.3 M-MgCl₂ Alcian blue staining (Zaniboni & Carinci, 1968).

In preliminary experiments no reactivity was detected with Alcian in the presence of 0.5–0.8 M-MgCl₂.

Since previous research with this material showed good consistency, both in vivo and in vitro, between histochemical data and biochemical determination, we felt entitled to use histochemical observations as indicative of quantitative changes [Pane et al. 1974; Carinci et al. 1975].

RESULTS

Three sets of experiments were performed: (a) cultures supplied with the same nutrient (E or CS) but changing culture dishes every 48 h to evaluate the suitability of our procedure; (b) cultures supplied with CS nutrient for only the first, second and/or third 48 h to determine the effects resulting from serum administration at different times; (c) cultures with actinomycin D added.

The results are based on 105 cultures and are summarized in the Table.

(a) Cultures in the same nutrient

E/E/E. At the end of incubation after 6 days in vitro, the epidermis comprised peridermal, intermediate and basal layers (Fig. 1), resting on an evident basement membrane which was strongly PAS reactive and Alcianophilic in 0.3 M-MgCl₂. The underlying mesenchyme was loose with large intercellular spaces containing material exhibiting a PAS positivity that was abolished by dimedone but not by diastase treatment, it stained strongly at 0.3 M and weakly at 0.025 M MgCl₂ with Alcian, and was partially removed by hyaluronidase digestion.

CS/CS/CS. By six days in vitro, the epidermis was 6–7 layered. Squamous layers were present, keratin made its appearance, and the periderm was still preserved (Fig. 2). The basement membrane was intensively PAS reactive and Alcianophilic in 0.025 M-MgCl₂. Dermal ground substance showed a strong PAS positivity, and Alcian staining, strong at 0.025 M and weak at 0.3 M-MgCl₂ (Fig. 7).

(b) Cultures supplemented with CS at different times

CS/E/E. Cultures administered with CS for the first 48 h and subsequently with E, showed at the end of incubation a three-layered epithelium (Fig. 3) resting on a strongly both PAS reactive and 0.025 M-MgCl₂ Alcianophilic basement membrane. Dermal ground substance was fairly PAS reactive, strongly Alcianophilic at 0.3 M and weakly at 0.025 M-MgCl₂ concentration (Fig. 8).

E/CS/E. Cultures supplied with CS for only the second 48 h, being maintained before and after in E nutrient, underwent typical epidermal cornification
Table 1. Epithelial histogenesis and dermal histochemistry of cultured skin

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Morphological data</th>
<th>Histochemical data</th>
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<tbody>
<tr>
<td></td>
<td>Three layered</td>
<td>Squamous layers</td>
</tr>
<tr>
<td>E/E/E</td>
<td>21</td>
<td>21</td>
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<tr>
<td>CS/CS/CS</td>
<td>18</td>
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<td>1</td>
</tr>
<tr>
<td>E/E/CS + Act</td>
<td>4</td>
<td>3</td>
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</table>

* CS indicates the chicken serum containing nutrient and E the chick embryo extract-containing nutrient (see Material and Methods); sequence indicates the type of nutrient supplemented to explants every 48 h; Act indicates actinomycin D. Alcian b – Alcian blue, Alcian y – Alcian yellow.

† Symbols: none; − − very weak; + weak; ++ increase in reactivity; ++++ strong; +++++ very strong.

‡ Before hyaluronidase treatment. § After hyaluronidase treatment.
FIGURES 1–6

Histological pictures of 6-day chick embryonic skin explants maintained *in vitro* for 144 h changing nutrients every 2 days as follows (hematoxylin-cosin).

Fig. 1. E/E/E. x 100.  
Fig. 2. CS/CS/CS/ x 1000.  
Fig. 3. CS/E/E. x 1000.  
Fig. 4. E/CS/E. x 1000.  
Fig. 5. E/CS + Act D/E. x 400.  
Fig. 6. E/CS/CS + Act D. x 600.
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FIGURES 7-10

Histochemical pictures of 6-day chick embryonic skin explants cultured for 144 h changing nutrient every 2 days as follows. (Sequential staining Alcian blue at 0·3 m-MgCl₂ and PAS; photographed with a Leitz 9520-55-90 green filter.)

Fig. 7. CS/CS/CS. x 240. Fig. 9. E/E/CS. x 240.
Fig. 8. CS/E/E. x 240. Fig. 10. E/E/CS + Act D. x 240.

(Fig. 4). The basement membrane was clearly PAS reactive and Alcianophilic in 0·025 m-MgCl₂. The mesenchyme contained a large quantity of intercellular substance that was definitely PAS positive, dimedone sensitive and diastase insensitive, intensively Alcian stained at 0·025 m and fairly at 0·3 m-MgCl₂.

Explants maintained in CS also for the third 48 h exhibited nearly the same morphological pattern (E/CS/CS).

In cultures examined at the end of CS supplementation (E/CS) the epithelium lacked any morphological sign of cornification and was composed of peridermal intermediate and basal layers. Mesenchyme showed a strong PAS-reactivity, a definite Alcian reaction at 0·3 m and a strong one at 0·025 m-MgCl₂.

E/E/CS. Cultures supplemented with CS during the last 48 h demonstrated a different epithelial behaviour, being three-layered or four- to five-layered with stratified squamous epithelium. Histochemically, fair PAS-positivity and a prevalent staining with Alcian in 0·025 m-MgCl₂ was observed (Fig. 9).
Cultures treated with actinomycin D

The antimetabolite was incorporated in CS medium and added either in the second 48 h to evaluate its ability to prevent epidermal cornification and the changes in histochemical features of mesenchymal ground substance; in the third 48 h to ascertain its action on the progression of serum-stimulated epidermal differentiation.

E/CS + Act D/E. Cultures so treated failed to differentiate (Fig. 5). The epithelium was well preserved and 3/4 layered (periderm, intermediate and basal cell). Mitotic figures were detectable. Necrotic mesenchymal cells appeared scattered through the dermis. The histochemical reactivity was reduced in comparison with the controls (E/CS/E), the ground substance exhibiting a weak PAS-positivity that was dimedone sensitive and diastase insensitive, and a moderate staining with Alcian at 0·3 and 0·025 m-MgCl₂.

E/CS/CS + Act D. As far as epithelial differentiation was concerned, no difference was observed in respect to the controls (E/CS/CS) (Fig. 6). On the other hand, dermal substance showed a reduced Alcianophilia, more pronounced at 0·025 m-MgCl₂, and a strong PAS-reactivity.

E/E/CS + Act D. Cultures exhibited a well preserved epithelium (peridermal, intermediate and basal cell layers). Histochemically, the mesenchyme was fairly PAS positive, and weakly stained with Alcian both at 0·025 and 0·3 m-MgCl₂ (Fig. 10).

DISCUSSION

The aim of the present work was to define the time course of developmental events taking place during serum-stimulated skin differentiation in vitro and to determine their dependence on the moment of serum administration using cultures in which media were changed sequentially.

Supplementation of skin cultures with CS or E nutrient for the whole incubation period, but transferring the explants every 48 h to another culture dish, resulted in the same epithelial differentiation and mesenchymal histochemical pattern as those previously described in cultures maintained in the same dish (Carinci et al. 1975). This indicated that methodology we used did not interfere with the in vitro differentiative processes and was therefore suitable for our experimental design.

Different epithelial and mesenchymal behaviour resulted from serum treatment depending on the time of its administration. When added for the first two incubation days, CS was ineffective in promoting the subsequent keratinization, in agreement with previous observations (Carinci & Simonelli, 1972). Histochemically, a greater accumulation of GAG than GP and a preferential concentration of CSAs as opposed to HA was detected in mesenchyme, i.e. the typical histochemical pattern of E maintained explants. Cultures supplied with CS during the second 48 h underwent epidermal keratinization. Correlatively,
the dermal ground substance exhibited changes in its composition characterized by an increased content of GP and an increased concentration of HA relative to CSAs. Inability of the first 48 h CS treatment to induce epithelial development was therefore due not to short exposure to serum but to unresponsiveness of skin cultures. Since recombinants experiments have conclusively shown that at this incubation time epithelium is already able to differentiate (McLoughlin, 1961b; Dodson, 1967), we can conclude that the lack of response rested with mesenchyme. CS administration for the last 48 h stimulated a certain degree of differentiation; squamous layers made their appearance in some cultures and, with continued in vitro maintenance, cornification was observed (unpublished data). Dermal substance showed a histochemical pattern intermediate between E and CS supplemented explants. It was apparent that in these conditions more time was required to accomplish differentiation. However, this finding demonstrated that after 5–6 incubation days epithelium and mesenchyme maintained their responsiveness to regulative stimuli and environmental factors.

Results from the actinomycin D experiments, based on differential sensitivity of the two tissues, epithelium and mesenchyme, to its action (Terao & Myaky, 1968; Zambonelli-Simonelli et al. 1972), helped to further clarify the time sequence of skin development in vitro. When added with CS in the second 48 h, it prevented keratinization without inducing epithelial damage (we observed mitotic figures) and also led to reduction and changes in composition of the dermal material (less amount of GP and HA in comparison to the controls). When added in the terminal incubation period, the antimetabolite did not interfere with epithelial differentiation, but did affect the amount of mesenchymal ground substance without altering the histochemical pattern qualitatively.

On the basis of the current and the previous data, we suggest the following time course of events in serum-stimulated skin differentiation in vitro.

In the first two days no serum-dependent effect was detected. Electron microscopy showed the same epithelial evolution (prominence of the Golgi complex and the granular reticulum, many ribosomes, a few desmosomes; i.e. ‘basal’ differentiation, Carinci et al. 1976), in explants supported either with CS or E. In addition, the two sets of cultures (CS or E) exhibited the same mesenchyme histochemical pattern characterized by a prevalence of GAG, mainly CSAs (Carinci et al. 1975). Finally, the previously observed ineffectiveness of actinomycin D administration during this period to affect keratinization (Zambonelli-Simonelli et al. 1972), indicates that serum induction takes place subsequently.

In the second 48 h the epithelium does not undergo relevant conspicuous morphological differentiation, as evaluated by histological and ultrastructural analysis. Instead, remarkable changes in dermal ground substance composition are detectable, the more obvious being the accumulation of GP and HA. Since cultures subsequently placed in the E nutrient, which is unable to stimulate epidermal differentiation, keratinized, the serum induction must take place in this period.
In the last period, 5–6 days *in vitro*, histological, histochemical and ultrastructural events of the keratinization take place. However, they have been previously programmed as demonstrated by actinomycin D experiments.

In conclusion, serum stimulatory factor(s) of epidermal differentiation act(s) in the central incubation period, inducing specific mesenchymal changes, both keratinization and mesenchymal development being affected by actinomycin D.

We are confident that extensive investigations during this period may throw some light on molecular mechanism involved in the regulation of skin differentiation.

These studies were supported in part by Italian CNR grant No. 76,01353,04.

**REFERENCES**


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(Received 10 August 1977, revised 12 December 1977)