Acta Dermatovener (Stockholm) 60: 223–228, 1980

EFFECT OF SERUM AND OXYGEN TENSION ON HUMAN SKIN ORGAN CULTURE: A HISTOMETRIC ANALYSIS

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Abstract. The addition of 20 % of calf or human serum to human skin culture, incubated in HEPES-buffered Eagle's medium in air atmosphere, considerably enhanced both epidermal cell migration (epibolization) and general viability of the epidermis, and increased the formation of new stratum corneum. The epidermal cell multiplication rate, as measured by thymidine incorporation was not, however, significantly increased by serum, nor was the disappearance of the granular cell layer counteracted. As an adverse reaction, serum was found to increase parakeratosis. The favourable effects of serum could be mimicked by adding to the serum-free basal medium a surplus of glutamine and 25 mM of sodium bicarbonate, and performing the incubations in an atmosphere containing 5 % carbon dioxide and 40 % oxygen. This significantly enhanced explant survival, and strongly increased the formation of new stratum corneum. In contrast to serum, this modification decreased the parakeratosis rate. Furthermore, a very significant increase in thymidine labelling was recorded. However, epibolization was much poorer than with serum. When serum was added to the 40 % oxygen incubation conditions, a strong epibolization effect was again obtained, but in other respects the results were not significantly superior to those obtained with serum-free 40 % oxygen conditions. It is concluded that most of the beneficial effects of serum in skin organ culture are unspecific and can be achieved in appropriate serum-free incubation conditions; in some respects even better results are obtained. Epidermal cell migration (epibolization) is an exception to this rule, indicating the existence of specific migration-enhancing serum factor(s).

Key words: Human skin; Organ culture; Oxygen tension; Serum; Histometric analysis

The skin organ culture is potentially an extremely valuable tool for the investigation of cutaneous physiology and pathology. A major drawback, is, however, the limited survival time and the early disturbance of normal tissue physiology of skin explants kept in a simple culture medium. Thus, significant numbers of scattered pyknotic spinous cells can be recorded already after 1 day of incubation, and confluent layers of necrotic spinous cells are encountered from the 5th day; the granular cell number starts to decrease on the 2nd day of culture (14).

Serum supplementation is an extensively used way of improving the viability of tissue cultures. In addition to increasing the general viability of skin organ cultures (3, 4), a specific stimulating effect of epidermal cell migration (epibolization) has been ascribed to serum (9, 10). The exact way(s) in which serum supplementation affects skin cultures is largely unsettled, however. In the present paper, the effects of serum on the survival and differentiation of the epidermis of organ-cultured skin explants have been analysed by using a quantitative histometric recording system, described earlier by us (14). As a comparison, the effects of glutamine surplus, carbon dioxide supplementation, and different oxygen tensions were investigated.

MATERIALS AND METHODS

Skin samples

Adult female skin was obtained at mastectomy operations from 6 donors, aged 35 to 79 years. Skin strips, about 2×10 cm in size, were taken immediately after operation, and transferred to culture medium. The subcutaneous fat was removed, and most of the dermal tissue trimmed away. Thereafter, pieces approx. 2.5 mm^2 in size were prepared.

Tissue culture

The organ culture method of Trowell (15) was used with minor modifications (14). As in our previous experiments (14) the standard incubation medium consisted of Eagle's minimal essential medium (modified) with Earle's balanced salt solution (Flow Laboratories Ltd.), buffered with 20 mM HEPES, and supplemented with 100 mg/l glutamine, 50 IU/ml sodium G-penicillin, and 50 μg/ml streptomycin sulphate. Atmospheric air was used as gas phase.

A medium containing, in addition, 25 mM sodium bicarbonate was used as a modification; this modified medium also contained a surplus of glutamine (600 mg/l). With this modified incubation medium, a gas atmosphere containing 5 % carbon dioxide, and either 95 % of oxygen, or 40 % of oxygen and 55 % of nitrogen was used.

In experiments designed to evaluate the effect of serum supplementation, 20 % of either newborn calf serum (Microbiological Associates, Bethesda, Md) or human serum

Table I. *The effects of serum at 10 days of incubation*

The mean values and standard deviations (S.D.) are given. Asterisk coding shows statistical significance of the differences in results obtained with and without serum (* denotes $p<0.05$; ** denotes $p<0.01$; *** denotes $p<0.001$)

Histometric parameter	Unit	No serum added	Calf serum 20%
Malpighian necrosis	% of section width	37.0±32.3	0***
New stratum corneum	μm	4.4± 4.4	11.0± 8.3***
Stratum corneum parakeratosis	% of sections analysed	86.5±34.7	100
Granular cells	Number	0	0
Vital spinous layer	μm	10.5±11.1	32.2± 18.0***
Vital spinous cells	Number	0.6± 1.0	2.9± 1.9***
Necrotic spinous cell layer	μm	42.0±27.0	5.0± 11.2***
Basal cell height	μm	5.8± 4.5	8.8± 2.1***
Epibolization	μm	0	888.3±847.8***

was used. Human serum was obtained from the University Hospital Blood Service in Turku.

Histological processing

Samples were harvested at the very start of the culture (control specimens), and on the third, fifth, seventh and tenth culture day, fixed in neutral buffered formaldehyde solution, dehydrated in alcohol and embedded in paraffin. With a microtome, one-fourth of the explant was cut and discarded, and thereafter about thirty perpendicular 7 μm sections were made, and stained with hematoxylin and eosin.

Histometric analysis

Using a quantitative histometric recording method, described in an earlier publication (14), the parameters listed in the Tables were recorded. Data from three independent experimental series were pooled. In each experimental series six explants were used for each incubation condition and each incubation period to be tested. From each explant, six sections were analysed. Every reported figure, thus, stems from a total of 108 individual measurements (3×6×6). The statistical significance of any differences between the results from the various incubation conditions was tested using Student's *t*-test.

Autoradiography

For autoradiography, skin explants were harvested on the 1st, 2nd, 3rd, 4th, 5th, and 6th culture day. The explants were incubated for 2 hours at 37°C in culture medium containing 5 μCi tritiated thymidine (Amersham, specific activity 28 Ci/mmol) per one ml of medium. Explants were washed twice with cold medium after the incubation, fixed in neutral formaldehyde, dehydrated in alcohol and embedded in paraffin. Five-μm thick sections were cut perpendicular to the epidermal surface, and coated by dipping with Kodak nuclear emulsion NTB 3 (Eastman Kodak Co., Rochester, N.Y.). The autoradiograms were exposed for 14 and 28 days at −15°C, developed in Kodak D-19 developer (Kodak Ltd., London, England) for 4 minutes and stained with hematoxylin and eosin.

For each explant the number of labeled nuclei were counted from over 4000 basal cells of the original epidermis (i.e. excluding the epibolus), using sections at least 30 μm apart. The combined data of two experimental series were used to calculate the means and standard deviations. Student's *t*-test was used for statistical analysis.

RESULTS

Effects of serum supplementation

The reference values for unincubated control specimens were: granular cell number 1.0±0.1, spinous cell layer 38.7±16.0 μm, spinous cell number 3.6±1.7, basal cell height 7.5±2.8 μm (data not shown in Table I). As anticipated, zero values were recorded for the other parameters, i.e. malpighian necrosis, new stratum corneum formation, parakeratosis, necrotic spinous cell layer, and epibolization. Table I gives data at 10 days of incubation, comparing plain culture medium with medium supplemented with 20% of newborn calf serum (CS). Incubations using 20% of human serum (HS) were carried out in parallel, but are not shown in Table I. All incubations were carried out in air atmosphere.

Both CS and HS supplementation had a very pronounced effect on the general viability of the epidermis. While in the absence of serum the average extent of malpighian necrosis was 37% of the section width, none could be registered in the presence of serum. No significant difference in the effects of HS and CS was observed with regard to this parameter or any other criteria for epidermal viability.

The process of keratinization was also clearly influenced by serum addition. A statistically very significant increase in the amount of new stratum corneum formation was noted both with CS (Table I) and with HS. In contrast, neither CS (Table I) nor

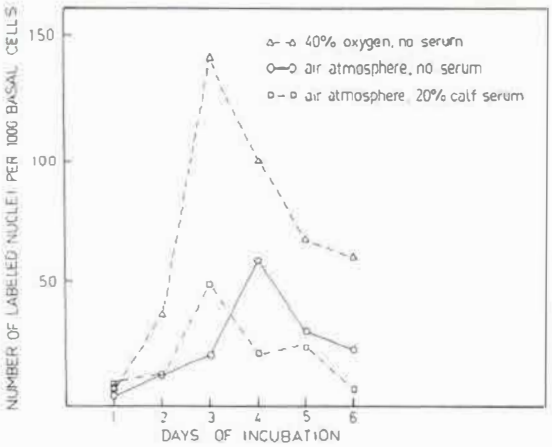

Fig. 1. Comparison of labelling indices in the original epidermis (i.e. excluding epibolus) of explants incubated in three different conditions. Each point represents the combined data for 2–5 explants. For each explant, over 4 000 basal cells were counted.

HS counteracted the disappearance of the granular cell layer by day 10. In fact, serum supplementation accelerated the disapperance of the granular layer from the 3rd day onwards and on the 5th day the difference in granular cell number between the serum-supplemented and the serum-free series was statistically significant ($p<0.05$; data not shown in the Table). An unfavourable effect of serum on the quality of the newly formed stratum corneum was also recorded, presenting as an increased incidence of parakeratosis both by CS (Table I) and HS. Although this increase did not reach statistical significance in the present series, the phenomenon has been reproduced at a statistically significant level in later experiments (unpublished).

A very impressive effect from serum supplementation was registered in epibolization. In the absence of serum, epidermal cell migration could be seen in the early days of incubation, but this epibolus later degenerated, and at day 10 none was evident (Table I). With serum supplementation, however, a steady increase in epibolus length was recorded during the first 5 (CS) or 7 days (HS). The epibolization was more prominent in explants supplemented with CS as compared with HS; from the 3rd day onwards, the difference was statistically very significant.

In contrast, serum supplementation did not enhance the rate of epidermal cell multiplication at any of the observed incubation intervals (Fig. 1), except at day 3, when the highest thymidine labelling index was obtained in the presence of serum. This maximum, however, did not supersede the maximal labelling index one day later in specimens incubated without serum.

Effect of different oxygen tensions

Table II compares the results from cultures using standard medium in air atmosphere with results from those using a medium surplussed with glutamine (600 mg/l), supplemented with 25 mM sodium bicarbonate, and incubated in an atmosphere of 5% carbon dioxide and either 40% or 95% of oxygen. All incubations were performed without the presence of serum.

The data for unincubated control specimens (not given in Table II) were: granular cell number 1.0+0.0, spinous cell layer 36.9+5.2 μm, spinous cell number 3.0+0.5, and basal cell height 11.1+1.6 μm; other parameters were negative. The basal values for day 10 of incubation in Table II (left column) correspond well to the basal data of Table I (left column), which were obtained under similar experimental conditions.

The specimens incubated in 40% oxygen showed a much better general viability than the air-incubated control specimens (Table II, malpighian necrosis, necrotic spinous cell layer). The differences are statistically very significant, yet the results are not as good as those obtained with serum supplementation (cf. Table I). The incubation conditions utilizing 95% oxygen were unfavourable as reflected in the extent of malpighian layer necrosis and of the height of necrotic spinous cell layer (Table II).

The incubation conditions utilizing 40% oxygen also very significantly increased the amount of new stratum corneum formation (Table II); this effect was even more prominent than that obtained with serum supplementation (cf. Table I). A significant decrease in parakeratosis formation was also observed. In contrast, the effect of 95% oxygen atmosphere was detrimental, decreasing stratum corneum formation, and greatly increasing parakeratosis (Table II). Neither 40% nor 95% oxygen incubation conditions could reverse the total disappearance of granular cells by day 10 (Table II). However, when the granular cell number was recorded on a day-to-day basis (data not shown), a slower reduction of the granular cell number could be recorded in the 40% oxygen incubated specimens compared with air incubated controls. Thus,

Table II. *Comparison of three incubation conditions at 10 days of incubation in the absence of serum*

The mean values and standard deviations (S.D.) are given. Each value is derived from 108 independent measurements. Asterisks in columns B and C indicate statistical significance as compared with data in column A. (Asterisk coding explained in the legend to Table I)

Histometric parameter	Unit	A Atmospheric air[a]	B Oxygen 40% CO_2 5%[b]	C Oxygen 95% C●₂ 5%[b]
Malpighian necrosis	% of section width	48.0±35.0	9.4±13.3***	68.4±31.6
New stratum corneum	μm	6.6± 8.1	28.7±10.6***	1.5± 5.4*
Stratum corneum parakeratosis	% of sections	8.20±38.0	64.4±33.8	100
Granular cells	Number	0	0	0
Vital spinous layer	μm	14.0±13.7	35.2±15.3**	5.5± 5.8
Vital spinous cells	Number	1.2± 1.6	3.5± 1.8**	0.3± 0.5*
Necrotic spinous cell layer	μm	29.8±18.1	2.7± 4.3***	40.3±14.5
Basal cell height	μm	8.7± 3.7	10.9± 2.0*	7.8± 2.1
Epibolization	μm	10.0±29.0	64.0±90.0*	12.0±37.0

[a] Medium buffered with 20 mM HEPES. Glutamine concentration 100 mg/l.
[b] Medium buffered with 20 mM HEPES and 25 mM sodium bicarbonate. Glutamine concentration 600 mg/l.

the granular cell count at day 7 was 0.11+0.26 for 40% oxygen incubation and 0.02+0.06 for air incubation; the difference is statistically significant ($p<0.01$).

Epibolization was recordable, albeit minimal both in the air-incubated and 95% oxygen incubated series (Table II). A statistically significant ($p<0.05$) increase in epibolus length was registered in the series using 40% oxygen. Compared with the results obtained in the presence of serum, this increase was very modest, however (compare Tables I and II). In contrast, a remarkable stimulation of the thymidine incorporation in the basal cell layer was recorded from the 2nd day onwards in the explants incubated in the defined medium in 40% oxygen atmosphere, as compared both with air incubation and serum supplementation (Fig. 1). The difference was statistically very significant ($p<0.001$) at days 2 and 3. Particularly at days 5–7, the proliferative activity of the epidermis in 40% oxygen atmosphere was also reflected in an acanthotic reaction of the spinous cell layer, giving a thickness of 65.8±22.5 μm and a cell count of 6.0±2.1 in the 40% oxygen series, compared with 29.1±25.5 μm and 2.2±2.3 cells in the air incubation series. At day 10, however, this acanthosis was partly abolished by excessive keratinization in the 40% oxygen incubated series (Table II).

Combination of serum and oxygen effects

To investigate the possible additiveness or oppositeness of the effects of serum supplementation and the 40% oxygen incubation conditions, 10-day incubations in 40% oxygen atmosphere (in concurrence with glutamine, bicarbonate and carbon dioxide supplementation) were performed both with and without the addition of 20% fetal calf serum. As expected, the addition of serum had a very favourable enhancing effect on epibolus formation, giving a migration of 234+140.0 μm with serum supplementation, compared with 39.0+66.8 μm without serum. This difference is statistically highly significant ($p<0.001$). However, the only other positive effect consisted of a slight enhancement of the general viability, recorded as a decrease in the amount of malpighian necrosis (8.0± 1.9% without serum, and none with serum) and in necrotic spinous cell layer height (0.4±1.4 versus none). Furthermore, serum supplementation gave an increased parakeratosis score (54.8±24.9% without serum and 90.8+ 14.6% with serum). Serum supplementation was not found to affect any of the other histometric parameters, i.e. stratum corneum formation, granular cell number, vital spinous layer thickness, spinous cell count, or basal cell height.

DISCUSSION

Many previous studies have reported on the influence of serum on skin organ cultures. Apart from measurements of epibolization, however, little quantitative data has been provided, and the reports are largely based on subjective descriptions, only. The present study compares, in a quantitative way

(14), the effects of serum supplementation and of different gas phases on the tissue survival, proliferation, migration and differentiation (keratinization) of the epidermis in human skin organ culture.

The common practice of supplementing culture media with serum primarily aims at enhancing the viability of the tissue. Our quantitative data confirmed the correctness of this generally accepted principle (Table I). Human serum and fetal calf serum were found to be equipotent in this respect. Our findings corroborate the statement of Curickshank and co-workers (6) that no tangible differences can be found between homologous, autologous or heterologous sera in the maintenance of adult skin in organ culture.

Another wellknown effect of serum is a stimulation of epidermal cell migration, resulting in epibolus formation (9, 10). This stimulation is also very clearly shown in our data (Table I). Not only was epibolus formation minimal in explants cultured in unsupplemented basal medium, but the epibolus was also particularly susceptible to degeneration, no epibolus remaing at the end of the culture period (10 days). Explants cultured in the presence of 20% serum showed a steadily increasing epibolization until day 5 or 7; thereafter a steady state was maintained. Marks & co-workers have reported autologous serum to be slightly more efficient than fetal calf serum in promoting migration in guinea-pig skin cultures (5), and homologous serum to be much more effective than fetal calf serum in the case of human skin (10). However, our explants displayed a more efficient epibolization in the presence of calf serum than with homologous (human) serum. The reason for this discrepancy is not clear, as our experimental conditions were similar to those used by Marks.

The available information on the effect of serum on the proliferative capacity of epidermis is inconclusive, partly because of variations in skin and serum sources, culture conditions, and length of incubation periods used by different investigators. Different serum concentrations have been compared in some studies, but the effect of serum supplementation on thymidine incorporation has been only rarely analysed (2, 9). Bertsch & Marks (2) measured thymidine incorporation in chick embryo epidermis incubated in Eagle's MEM with or without 15% fetal calf serum. While an approximately two-fold incorporation was noted with serum supplementation at 24 and 48 hours, unsupplemented conditions gave a slightly higher count at 72 hours. Levine (9), culturing adult human skin in Eagle's MEM with or without 10% human serum noted no differences at 5 days of incubation, while the unsupplemented series gave a higher incorporation at 8 days; with prolonged culture times (12 to 19 days) an increase in thymidine incorporation was noted in the serum-supplemented series, while the reverse was true for the unsupplemented series. In the present investigation, the effect of serum supplementation on the thymidine labelling index was recorded from day 1 to day 6. While serum supplementation shifted the maximum of incorporation from the 4th day of incubation to the 3rd, the overall effect of serum was insignificant (Fig. 1).

An unfavourable effect of serum on the quality of keratinization, presenting as increased parakeratosis, has been noted by earlier investigators (4,9). In the present quantitative study, the parakeratosis-increasing effect of serum was demonstrable under two different conditions, i.e. in plain basal medium in air atmosphere, and in enriched basal medium (glutamine 600 mg/l, sodium bicarbonate 25 mM) in 40% oxygen and 5% CO_2 atmosphere. Human and calf serum were found to behave similarly. Analogously, serum supplementation could be shown to accelerate the disappearance of the granular cell layer. The basis for these untoward effects of serum on the keratinization process is unknown, but of interest in this context is the observation made by Levine (9) that high molecular weight constituents of human serum increased the extent of parakeratosis in human skin explants.

The present study clearly demonstrates that modifications of the defined culture conditions can also, in a significant way, alter the vitality, proliferation, migration and differentiation of epidermis. By modifying the defined culture conditions, the favourable effects of serum on tissue survival and rate of stratum corneum formation were mimicked and, in fact, a more advantageous effect on the proliferation rate and on the quality of the keratinization was obtained. The most important single variable was the oxygen tension. While an increase in the oxygen level from that of air (i.e. about 20%) to 40% proved very beneficial, a level of 95% was clearly detrimental. These findings agree very well with those of Raeven & Cox (12, 13). They found that an atmosphere with 95% oxygen sharply reduced the survival of human skin explants, while one with

95% air was found to be better (12); maximal mitotic activity was noted with an oxygen tension of 40% with the activity falling off on both sides of this point (13). Melcher, however, found the optimal oxygen concentration for the survival of rat skin to be 70% (11), and Trowell considered 95% oxygen to work as well as air atmosphere (15). Bergenholtz, on the other hand, compared 20, 50 and 95% oxygen concentration in cat palatal mucosa organ culture; 50% was found to give the best survival, and 20% the worst (1). The discrepancies of these results are somewhat disturbing, but may be due to differences in preparation and incubation techniques, or to species variation. In our laboratory, the incubation conditions using 40% of oxygen have continued to give consistently good results in human skin organ culture.

The role of glutamine surplus and the presence of the bicarbonate–carbon dioxide buffer is difficult to evaluate. These were included on the basis of recommendations found in the literature (7, 8). Eagle and co-workers originally demonstrated the essential role of glutamine for the growth of cultured cells; omission of glutamine arrested cell duplication, the optimal concentration of glutamine for different cell lines differing by one order of magnitude (7). In our own preliminary experiments (unpublished), an elevation of the glutamine concentration from 100 to 600 mM enhanced the growth, vitality, and differentiation of the epidermis. The addition of the bicarbonate–carbon dioxide buffer system slightly enhanced the rate of keratin layer formation, but no other effects were recorded. While the value of the glutamine surplus and the bicarbonate–carbon dioxide buffer in the final culture system is debatable, we have adapted as our routine procedure the incubation condition of Eagle's MEM with 600 mg/l glutamine, 20 mM HEPES, and 25 mM sodium bicarbonate, in an atmosphere of 40% oxygen, 5% CO_2, and 55% nitrogen.

The present investigation sheds some light on the specificity of the serum effects on skin culture. The effects on general viability and on the rate of keratin formation seem to be rather unspecific, as they can be largely reproduced by modifications of the defined incubation conditions. In contrast, the dramatic effect of serum on epibolization can only feebly be mimicked by such modifications. This finding points towards the existence of a specific migration-enhancing factor(s) in serum, as has been postulated by previous investigators (9, 10).

ACKNOWLEDGEMENT

This work was supported by the Emil Aaltonen Foundation.

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Received October 27, 1979

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