Effect of mechanical pressure on c-fos and on the mitotic activity of epidermal cells

Roumen Tsanev, Julia Yaneva, Kalina Vaptzarova and Dimitar Markov

Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

Received: 9 May, 1996

Key words: epidermis, c-fos, cell cycle, cAMP

A dosed mechanical pressure of 12.5 kg/cm² applied for 1 min on depilated mouse skin did not cause cellular death or visible alterations of the cellular ultrastructures. However, it had a strong effect on the mitotic cycle of the epidermal cells—stimulating the cells to enter the mitotic cycle and temporarily blocking the G1 → S transition. This effect was strictly limited to the pressed area of the skin. The proto-oncogene c-fos was induced within the first 2 min following application of the pressure. The level of c-fos mRNA showed two peaks during the next 24 h. The first slight peak was preceded by a rapid increase in the cAMP level in the pressed skin, the second—by a fall in the cAMP concentration. A model is suggested to explain the observed effects by reversible functional damage of the cellular membrane affecting the enzymes maintaining the steady state level of cAMP.

The proto-oncogene c-fos belongs to immediate early genes and can be induced by a great number of different biologically active agents—growth factors, hormones, various organic and inorganic compounds, UV-irradiation, etc. This oncogene was found to have pleiotropic activity, involved in such mutually exclusive processes as cell proliferation and differentiation (see [1]), while its deregulation was associated with malignant transformation [2].

c-fos was reported to be expressed in all cell layers of the epidermis [3–6] and was found to be induced by many more or less specific agents [1]. It was of interest to see whether it could be induced by such a nonspecific factor as mechanical pressure on the epidermal cells which are often subjected to mechanical forces and are actively engaged both in cell proliferation and in apoptosis.

In the present study we have found that a non-necrogenic mechanical pressure on the mouse skin disturbs immediately the steady state level of cAMP, initially leading to its strong increase followed by a decrease. These fluctuations in cAMP concentration were followed by two peaks of c-fos induction and a complex effect on the mitotic activity of the epidermal cells strictly limited to the pressed area.

MATERIALS AND METHODS

Animals and treatment. The skin on the back of albino mice (20–25 g body weight) was depilated by treatment with barium sulphide two days before the experiment. With the animals under ether narcosis, a strictly dosed mechanical pressure of 12.5 kg/cm² was applied for 1

*a Author for correspondence. Tel: +(359-2) 73 74 78; Fax: +(359-2) 72 35 07.
min on a fold of deplated skin by using a simple device [7] (Fig. 1) producing two pressed skin areas of 1 cm² each. The pressed area was marked so that it could be precisely separated from the adjacent control skin.

**Histological procedures.** Samples of pressed and adjacent skin were fixed in ethanol/40% formaldehyde/acetic acid (17:2:1, by vol.) and in ethanol/acetic acid (3:1, v/v). Paraffin sections 7 μm thick were prepared and stained with Urona's methyl green/pyronine.

**Electron microscopy.** Pressed and adjacent intact skin samples were fixed for 1.5 h at room temperature in 0.1 M cacodylate buffer, pH 7.4, containing 4% formaldehyde (freshly prepared from paraformaldehyde) and 2.5% glutaraldehyde. The material was carefully washed in the same buffer and postfixed for 2.5 h in 2% OsO₄ dissolved in the same buffer. After dehydration in ethanol it was embedded in Epon 812. Ultrathin 90 μm sections were stained with uranyl acetate and lead citrate. They were studied in a JEM 100B electron microscope at 80 kV. The pressed skin was used either immediately or 1 h after application of the pressure.

**Estimation of arrested metaphase plates.** The number of cells arrested in metaphase during a 6-h period was estimated. To this end, 6 h before being killed the mice were injected with colcemide (2 μg/g body weight). The total number of epidermal cells and of metaphase plates per microscopic field along the section length (magn. 10 x 100) were determined at different times after application of pressure as an average from counting of 10–20 sections. The figures were converted to metaphase plates per 1000 cells.

**DNA synthesis.** Beginning from the 6th hour after application of pressure, every 2 h groups of 3 mice were injected intraperitoneally with 50 μCi/mouse of [³H]TdR (Amersham, 5 Ci/mmol). The pressed and two adjacent skin samples were frozen in liquid nitrogen and weighed on a precision "OHAUS" balance. They were ground to powder, the acid-soluble material was extracted with 0.2 M HClO₄ in the cold and its radioactivity measured in a Beckman LS 1801 counter. The remaining material was hydrolysed in 1 M KOH for 18 h at 37°C. After neutralisation with 1 M HClO₄ the RNA-containing supernatant was discarded, the remaining material washed with 1 M HClO₄ and DNA extracted with 1 M HClO₄ at 70°C. Its quantity was determined by the two-wavelength method [8]. The specific radioactivity of DNA was calculated as ³H radioactivity per mg DNA corrected for the radioactivity pool (³H radioactivity of the acid-soluble extract per mg tissue) accepting that within the small variations of the pool (between 35% and 10%) the incorporation of the precursor is proportional to the pool size.

**Isolation of RNA.** The skin samples were frozen in liquid nitrogen, ground to fine powder, suspended in cold saline solution and immediately mixed with an equal volume of saline-saturated phenol, pH 6.0. After 2 h of vigorous shaking the aqueous layer was separated by centrifugation and deproteinized with phenol (twice), phenol-chloroform (1:1, v/v) and chloroform. RNA was precipitated with three volumes of ethanol at -60°C and used within the next two days.

**Estimation of c-fos mRNA content by dot hybridization.** RNA, 20 μg, dissolved in 200 μl of 16.5% formaldehyde was heated for 20 min at 65°C and cooled on ice, then 30 x SSC was added to a final concentration of 6 x SSC. The samples were applied onto Millipore 0.45 μm membranes. After being dried under vacuum at 80°C for 2 h, the RNA dots were hybridised [9] with a DNA probe ³²P-labelled by nick-translation (sp. activity 1–3 x 10⁸ c.p.m./μg DNA). The probe comprised a fragment of the human v-fos gene [2] from position 1504 to 2800

---

**Fig. 1. Device for application of a dosed mechanical pressure.**

W is a sliding weight fixed on the rod R. Its position determines the pressure p applied on the skin-fold S, \( p = \frac{W}{T} \).
cloned in pGEM1. This probe hybridised in Northern blot with a band located between 28S and 18S ribosomal RNAs, corresponding to the localisation of c-fos mRNA, as shown in many studies (e.g. [45, 46]). After hybridization the filters were washed under stringent conditions, air-dried and counted in the Beckman 1210 Scintillation Counter. The dots were also autoradiographed and their blackening measured with the Quick Quant II Autoscaner (Helena Lab. Co., Texas, U.S.A.). For each time point 5–6 mice were used. In view of the argument that the 5 times repeated CAAAA motif in the above defined fragment may lead to a non-specific signal [10], we also used for hybridization a fragment with this repeat excised.

**Determination of cAMP**. This was performed on male animals (20–25 g body weight) during the same season and under standard conditions of feeding and room temperature. For each time point three mice were used, thus obtaining six pressed and six adjacent skin samples. The samples were immediately frozen in liquid nitrogen, weighed as above and homogenised with the “Polytron PTA 10s” homogeniser in ice-cold 0.5 M HClO4 containing 25% ethanol. After centrifugation the supernatant was neutralised to pH 7.0 with 0.5 M KOH and lyophilised. The pellet was used for protein determination. cAMP was determined in the lyophilised material according to Gilman [11] with the Amersham Cyclic AMP 3H assay system. Preliminary experiments showed that the extracts did not contain substances interfering with the cAMP-binding protein and the recovery of cAMP was 75%. The alkali-soluble protein was determined in the pellet by the biuret reaction after boiling in 1 M NaOH [12]. The concentration of cAMP was calculated as pmoles/mg protein.

**Statistical significance** of all results was estimated according to Student’s t-test.

RESULTS

State of the epidermal cells

The mouse epidermal cells were found to survive one-minute pressures of up to 15 kg/cm². In the present experiments we have used a pressure of 12.5 kg/cm² applied for 1 min. All cells were preserved alive after this treatment as judged by histological examination of the skin sections. As found long ago [13, 14], an early sign of irreversible cell damage can be easily detected by the use of Unna’s methyl green-pyronine staining of cells fixed in ethanol/acetic acid. An instant strong increase of the cytoplasmic staining with pyronine and a pyroninophilia of the nuclei is observed in irreversibly injured cells. Later, the pyronine staining of the cytoplasm of dead cells disappears due to total degradation of the cytoplasmic RNA and the nuclei become pycnotic. A later sign of cell death is the accumulation of leukocytes around the dead cells, a well known consequence of necrogenic injury. A careful examination of the skin sections showed that in the present experiments no such events took place.

In addition, electron microscopic studies performed immediately and 1 h after application of pressure did not reveal any differences between the cellular ultrastructures of the pressed and the control epidermis — plasma membranes, mitochondria, ergastoplasm with ribosomes, nuclear membrane and chromatin of the cells in the pressed skin did not differ from those of the control skin (not shown).

**Number of epidermal cells arrested in metaphase**

Treatment with a pressure of 12.5 kg/cm² for 1 min significantly increased the number of colcemide-arrested metaphase plates estimated from 30 pressed areas for each time point. This figure increased significantly 18 h after application of pressure, showed a maximum at the 24th hour (an increase of 444%) and still a 368% increase at the 30th hour (Table 1). The normal value was restored after 36 h (Figs. 3 and 4). All metaphases were localised in the basal layer (Fig. 2). It is important to note that the number of cells arrested in metaphase in the adjacent epidermis was not affected, showing a mean value of 6.2 ± 0.4 per thousand calculated from 60 control areas.

**DNA synthesis**

The incorporation of [3H]TdT in the control skin increased linearly during the 20 h period studied. In the pressed skin the increase was also linear but the curve showed two segments with different slopes — an initial slope slightly lower (by 15%), and a second segment with a slope 67% higher than that of the control curve.
Table 1

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Pressed epidermis</th>
<th>Adjacent epidermis</th>
<th>Increase (%)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>13.3 ± 1.0</td>
<td>6.1 ± 0.5</td>
<td>118 ± 8.9</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>24</td>
<td>27.2 ± 2.3</td>
<td>5.0 ± 0.9</td>
<td>444 ± 37.5</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>30</td>
<td>31.4 ± 2.6</td>
<td>6.7 ± 0.8</td>
<td>368 ± 30.5</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>36</td>
<td>7.9 ± 1.0</td>
<td>6.8 ± 1.0</td>
<td>16 ± 2.0</td>
<td>-</td>
</tr>
</tbody>
</table>

(Fig. 5) indicating a correspondingly higher synthesis rate. On expressing the changes of the \[^{3}H\]Tdr incorporation in the pressed skin as percentage of the data for the control skin, it was found that there was an initial rapid suppression of DNA synthesis followed by its increase after about 10 h (Fig. 6). The period of decreased DNA synthesis varied in different mice from 6 to 16 h with a mean value of 10 ± 1 h, as estimated from experiments with 45 mice. The figure of 10 h corresponds to the period of decreased rate of \[^{3}H\]Tdr incorporation in Fig. 5.

Expression of c-fos mRNA

The mechanical pressure quickly induced the c-fos gene mRNA. As early as 2 min after application of pressure the mRNA content showed a small but statistically significant increase of 17.2 ± 4.5% (P = 0.011). It is of interest that the time course of the elevated c-fos mRNA content showed two peaks — an initial rapid increase of 103 ± 9.6% (P < 0.001) lasting for the first 3 h and a second stronger increase of 305 ± 13.4% (P < 0.001) at the 6th hour after application of pressure. Then the mRNA content steadily decreased, approaching its initial level at the 24th hour (Fig. 4). The presence of two peaks of increased c-fos mRNA content was statistically significant.

Cyclic AMP level

The control skin samples contained 5.54 ± 0.35 pmoles of cAMP/mg of alkali-soluble protein. In the pressed skin its mean value was 14.1 ± 1.7 pmoles/mg protein (a 168 ± 39% increase, P < 0.005) almost immediately after application of the pressure. Ten minutes later it was still increased by 129 ± 31% (P < 0.004). During the first hour it fell below the normal level to a mean value of 2.8 ± 0.4 pmoles/mg protein (a 56% decrease, P < 0.008). The initial value was restored at the 6th hour (Fig. 4). The 65 ± 35% increase at the 24th hour was not statistically significant (0.05 < P < 0.1).

DISCUSSION

The mechanical pressure used in the present experiments did not kill the epidermal cells. This was proven by several observations during a 24 h period after application of pressure: a. Absence of morphological changes under the light microscope; b. Absence of changes in staining of the cytoplasm and nuclei with
microscope; d. Absence of leukocyte infiltration in the area of the pressed skin.

The data obtained have shown that such a non-necrogenic pressure causes the following statistically significant changes in the pressed area of the skin:

1. An almost immediate fluctuation in the level of cAMP revealed first as a peak of a 168% increase, followed after 1 h by a 56% decrease with respect to the initial level (Fig. 4).

2. A biphasic induction of the c-fos mRNA with a first peak of about 100% increase lasting 3 h and a second stronger peak of a 300% increase 6 h after application of pressure (Fig. 4). A biphasic curve of c-fos induction has also been observed after UV-irradiation [15].

3. A stimulation of cell proliferation revealed as an increased entry of epidermal cells from the basal layer into G1 (Figs. 2 and 5).

4. A temporary block of the G1 → S transition with a mean duration of 10 h (Fig. 6).

As a result of the last two effects on the mitotic cycle an about 450% increase in the number of epidermal cells arrested in metaphase by colcemide was observed 24 h after application of pressure (Fig. 4 and Table 1). This was partly due to a real stimulation of cell proliferation (about 50%), and partly to a synchronisation of the mitotic cells by the G1 → S transition block. As seen in Table 1 the number of cells entering the mitotic cycle during a 6 h period of colcemide block was fairly constant in the control epidermis with a mean value of 6.2 ± 0.4 per thousand cells. Thus, even a block of 16 h (the maximal value observed) could be responsible

Unna's methyl green-pyronine; c. Absence of ultrastructural changes under the electron microscope.

Fig. 3. Colcemide-arrested metaphase plates in the pressed and adjacent mouse epidermis.
The columns show the number of metaphase plates (ordinates) estimated per 1000 epidermal cells for each successive microscopic field (abscissae) at a magnif. 10 × 100 after a six-hour colcemide block measured at different times after a pressure of 12.5 kg/cm² was applied for 1 min. (The bars delimit the pressed area)

Fig. 4. Time course of changes in c-fos mRNA (●), cAMP (■) and of colcemide-arrested metaphase plates (▲) in the epidermis after a pressure of 12.5 kg/cm² was applied for 1 min.
Abscissa, time; ordinate, percentage changes in the pressed area with respect to the adjacent control skin. Vertical arrows indicate the standard error of the mean.
were the result of a direct effect on the cells. Most probably this was due to a reversible damage to the cellular membranes. This is indicated by their increased permeability — the acid-soluble radioactive pool in the pressed area was increased up to 35% during the first hours after application of pressure.

A reversible damage to the cell membrane may provide a reasonable explanation for the very rapid changes of cAMP concentration in the pressed skin. A functional damage to the cell membrane should affect the mitogenesis-related ion channels [16] and will disturb the normal relationship between the membrane-linked G-proteins and their effectors. The latter include the enzymes of the cAMP turnover — adenyl cyclase and a phosphodiesterase [17, 18]. If the activity of these two enzymes is differentially affected by the pressure-induced changes in the membrane, a fluctuation in the steady-state level of cAMP will rapidly occur.

According to recent literature data, changes in the cAMP concentration can be causally linked to the induction of the c-fos mRNA. It has been shown [19–21] that the c-fos gene is induced by the trans factor CREB when the latter is phosphorylated by the cAMP-dependent protein kinase (PKA). This explains why an increased cAMP concentration has been found to induce this gene in a number of different cell types [22–32]. Our data show that the same holds true for the epidermal cell — the highly increased cAMP level during the first minutes after application of pressure could explain the appearance of the first peak of increased c-fos mRNA content.

The induction of c-fos gene is usually of a very short duration due to a process of autorepression exerted by the gene product Fos when its C-terminus is phosphorylated by the same cAMP-dependent kinase [33, 34]. In our experiments the subsequent 56% fall in the concentration of cAMP should lower the phosphorylation of Fos leading to a release of the autorepression. This may well explain the second peak of c-fos mRNA increase which followed the decrease in cAMP level. The activity of the gene reached again its initial value when the concentration of cAMP returned to normal (Fig. 4).

c-fos is one of the first proto-oncogenes induced in some cell types stimulated to prolifera-
rate [1]. With respect to the epidermal cells it has been concluded that c-fos expression is associated with their differentiation and apoptotic death, and has little effect on regulation of cell proliferation [35–37]. However, other data show that c-fos mRNA and the Fos protein are expressed in equal amounts in all layers of the epidermis [3–6]. Our results show that, like in other cell types, c-fos expression precedes cell proliferation of the epidermal cells when they are induced to proliferate by mechanical damage. There is no reason to reject a causal relation between the two processes. On the other hand, recent data show a strong similarity between the processes mediating cell proliferation and apoptosis [38–40]: in cells entering G1 and in cells subjected to apoptosis the same sets of early genes controlling the cell cycle are activated. Thus, activation of c-fos may be involved in both processes.

Of great interest is also the G1 → S block induced by the mechanical pressure. Such a block is known to result from DNA breaks leading to activation of the p53 pathway [41, 42]. In our case we have no evidence either in favor or against such a mechanism, since it has been stated that even a single double-strand break in DNA may induce p53 (see [43]). Another mechanism which we would like to suggest is again the fall in cAMP concentration during the 6 h period after application of pressure. The G1 → S block is known to be mediated by the product of the retinoblastoma gene pRB and is normally prevented by a cdk1/cyclin D1 hyperphosphorylation of pRB ([44] and refs. therein). The phosphoprotein cyclin D1 was also found to be phosphorylated by the cAMP-dependent PKA [44]. It may be speculated that the pressure-induced fall in cAMP may prevent the phosphorylation of cyclin D1 by PKA, thus allowing pRB to block the entry of cells into the S phase. Further experiments are needed to study this possibility.

In conclusion, our data show that a reversible damage to the epidermal cells can induce fluctuations in the concentration of cAMP which appear to be an important membrane-linked signalling mechanism affecting the cellular oncogene c-fos and the mitotic cycle of these cells.

REFERENCES


Fos protein suggest important role in the transition from viable to cornified cell during keratinization. Development 111, 253–258.


44. Sewing, A. & Muller, R. (1994) Protein kinase A phosphorylates cyclin D1 at three distinct sites within the cyclin box and at the C-terminus. Oncogene 9, 2733–2736.
