Contribution to the Study of Excision Repair Following UV Irradiation in Mammalian Cells Cultivated in Vitro

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The absence of pyrrolidine dimer excision after UV irradiation in rat cell line was confirmed. The possibility of excision repair system depression by acidification of medium with H$_3$PO$_4$ in HeLa cells has been proved. The results obtained by two different methods of deproteinization and DNA isolation are in good conformity.

Key words: DNA, UV irradiation, repair mechanism, pyrimidine dimer.

UV irradiation is known to injure cells by producing photoproducts (mainly pyrimidine dimers); such lesions can be removed from the damaged DNA [3]. Human and many other mammalian cells possess the so called excision repair ability. The first results on Chinese hamster and mouse L cell lines suggested, however, that the UV photoprodutcs are not excised from the damaged DNA [10, 19]. Some recent papers bore evidence to the fact that most rodent cell lines are not able to provide excision repair [4]. There were only a few exceptions to this rule for example — mouse ascites and Chinese hamster CHEF 125 and CHO cells [4, 12].

However one of the latest studies by Ben-Ishai and Peleg [1] has brought a new finding in mouse cells. The excision of pyrimidine dimers (TT) has been lost at highly passages but the primary cultures and earlier passages are able to excise UV lesions.

There are possibilities to explain the loss of the UV endonuclease activity (and therefore excision activity): 1. genetically fixed loss of this enzyme, 2. blocking its production, 3. blocking its function by some inhibitory factors.

We have taken an interest in the last possibility in our experiments, though it is known that excision ability is very resistant to different physiological influences [15]. The fact, that rodent cells, in spite of having no dimer-excision ability, possess a low level of unscheduled synthesis seems to be also interesting [8, 14]. We have found the excision inhibitory influence of acid extracellular environment at excision proficient human HeLa cells. Besides the absence of excision at rat LEF cells in higher (126th) passage has been confirmed. Analytically we have verified the susceptibility of two methods of deproteinization; enzymatical (lysosym-pronase) and chemical (chloroform-isoamylalcohol) methods give the same results but the first of them seems to be more elegant.
Material and Methods

Cell lines and cultivation. LEF cells were derived from embryonal tissue of Lewis White rat strain and at the time of experiment were in 12th passage. They were seeded in amount of $3 \times 10^6$ in Petri dish (100 mm diameter) and cultivated in a CO$_2$ thermostat in minimal Eagle’s medium (MEM) (Institute of Sera and Vaccines, Prague) with 10% fetal calf serum and 10% TPB (Difco).

HeLa cells were cultivated in the usual manner in Roux flasks in basal Eagle’s medium (BEM) with 6% calf serum. In the experiments we seeded $1.5 \times 10^6$ cells on a Falcon plastic Petri dish of a diameter of 100 mm. The culture medium was buffered with an inorganic buffer system in such a way that a thermostat without CO$_2$ atmosphere could be used. We utilized no CO$_2$ atmosphere to be able to measure readily the pH of the medium by which we took our orientation in the samples acidified with H$_3$PO$_4$.

Labeling, irradiation and taking of samples. To each Petri dish we added thymidine-6-$^3$H (Institute for Research Production and Uses of Radioisotopes, Prague), spec. activity 22 Ci/mM, in such a way that the concentration was 10 μCi/ml for LEF cells and 5 μCi/ml for HeLa cells respectively. After labeling for 24 hours, the cells were irradiated with ultraviolet light (6.3 ergs/mm$^2$/s). The dose was changed according to the given conditions of the experiments. As a source a germicidal Philips TUV 15W lamp was used. Intensity was measured on a IL 254 (International Light, USA) dose-rate-meter.

Samples were taken either immediately after irradiation, or at indicated time intervals. Nonradioactive medium had been in some cases acidified with H$_3$PO$_4$ at postirradiation incubation. pH was measured on an OP-205 pH-meter (Radelkis, Budapest, Hungary) with the aid of a glass and calomel electrode.

Cells were removed from the dishes by “dry trypsinization” (with the aid of a minimum amount of trypsin — appr. 0.5 ml). They were then suspended in 10 ml NET buffer and centrifuged at 2000 rpm at a temperature of 0 °C. The sediment was resuspended in 0.5 ml NET buffer diluted in a 1 : 10 ratio. This suspension was rapidly refrigerated to —80 °C in a methanol-dry ice mixture. Samples were subsequently transferred and stored at a temperature of —20 °C until used.

Isolation of DNA. Samples used in determining thymine dimers were deproteinized enzymatically or chemically.

a) In the enzymatic deproteinization we added to the samples lysozym (Calbiochem, USA) (0.4 mg per sample) for 15 min. at 30 °C. Then 0.2 mg pronase (Calbiochem, USA) was added to each sample, with subsequent incubation for 30 min in a water bath at 60 °C [7].

b) In the chemical deproteimation the cells were treated by sodiumlaurylsulfate for 15 min at 60 °C (British Drug Houses, England), final concentration 1 mg/ml. Deproteinization was carried out with chloroform-isoamylalcohol 24 : 1 mixture in a ratio to the sample of 1 : 1 [12]. The mixture was after vigorous shaking in a laboratory mixer subsequently centrifuged at 3000 rpm at room temperature; the supernatant was used for the determination of thymine dimers.

Deproteinized samples obtained by either methods were cooled to 0 °C and the DNA precipitated with 10% TCA at the above mentioned temperature and the precipitate was centrifuged and dried with 2 ml of 96% ethanol.

Determination of thymine dimers. Dried samples were hydrolyzed with 0.2 ml 98% HCOOH at 175 °C for 60 min. Hydrolysates were divided for dimer estimation.
by two-dimensional paper chromatography on Whatman No 1. For the developing, in the first dimension n-butanol : water = 86 : 14 (v/v) and in the second saturated ammonium sulphate : 1 M sodium acetate : isopropanol = 40 : 9 : 1 (v/v/v) mixtures as described by CARRIER and SETLOW [3] and modified by SHLAES et al. [17], were used. The regions of thymine and its dimers on the chromatograms were cut in 5×1 cm strips and shaken in dioxan liquid scintillator with water (5 ml + 0.3 ml) for 30 min. Radioactivity of strips was measured on Liquid Scintillation Spectrometer Triacarb 3375 (Packard, USA). The ratio of dimers to thymine could then be calculated.

**Results**

**Absence of TT excision in LEF cells.** As can be seen from Fig. 1 the rat LEF cells are unable to excise thymine dimers. No excision of TT was observed after 48 h cultivation at the used dose 75 and 3000 ergs/mm$^2$ respectively.

**TT excision and its depression in HeLa cells.** HeLa cells show evident excision of TT in normal medium after 12 hours (Fig. 2). The excision stops in the medium acidified with $\text{H}_3\text{PO}_4$. This environment has no significant influence on the radioactive background in TT region of unirradiated samples (Fig. 2 and legend to Table 2).

A comparison of the two methods of deproteinization is shown in Table 1. No significant differences were observed in the results. In cells which became detached from the cultivation surface (dead or dying cells) the level of excision is the same as in cells attached to the bottom (Table 2).

![Fig. 1. Thymine dimer content during UV postirradiation cultivation in rat cells. Cells were prelabeled with thymidine-6-\(^3\)H for 24 hours and then irradiated with given doses of ultraviolet light. At indicated time intervals the content of TT was estimated radiochromatographically. Dotted line shows the radioactive background on the chromatograms (unirradiated samples).](image-url)
Fig. 2. The influence of $\text{H}_3\text{PO}_4$ on the excision of thymine dimers from UV irradiated HeLa cells. Cells were cultivated and prelabeled with thymidine-6-$\text{H}^3$ prior to the irradiation. After irradiation with 300 ergs/mm² they were cultivated in normal BEM (pH 7.15) and in BEM acidified with $\text{H}_3\text{PO}_4$ (pH 5.83). Thymine dimers were estimated immediately or 12 hours after irradiation. The initial content of $\text{TT} = 100\%$, // // // // — radioactive background.

Discussion

We have proved that excision repair system of HeLa cells can be depressed by acidification of cultivation medium with $\text{H}_3\text{PO}_4$. Two different methods of deproteinization and isolation of DNA from UV irradiated HeLa cells have been used. The results obtained by both methods are in a good conformity and in agreement with results in human cells published earlier [5, 16].

It is true that the enzymes responsible for the excision of photoproducts — endonuclease and exonuclease — have, at least in the case of strain Micrococcus, their pH optimum in the neutral region [6] and in the case of HeLa cells in alkali region [2]. It is likewise beyond doubt that the pH in the cytoplasm may very considerably (even in physiological conditions in mammals between 5.0 and 7.8). Intra-

Table 1. Comparison of two isolation methods in TT estimation in HeLa cells

<table>
<thead>
<tr>
<th>Methods</th>
<th>Time after UV</th>
<th>CPM</th>
<th>% TT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TT region</td>
<td>T region</td>
</tr>
<tr>
<td>Pronase</td>
<td>0 h</td>
<td>355.5</td>
<td>333 838</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0 h</td>
<td>351.7</td>
<td>332 674</td>
</tr>
<tr>
<td>Pronase</td>
<td>12 h</td>
<td>459.9</td>
<td>422 857</td>
</tr>
<tr>
<td>Chloroform</td>
<td>12 h</td>
<td>423.7</td>
<td>404 471</td>
</tr>
</tbody>
</table>

After 24 hour cultivation in the presence of thymidine-6-$\text{H}^3$ the cells were UV irradiated with a dose of 300 ergs/mm² and TT were estimated immediately (time zero) and after 12 hour cultivation at pH 5.83. Two deproteinization procedures were used: enzymatical with lysosyme-proprase and chemical with chloroform-isoamylalcohol. Dimers were estimated in attached cells (see legend to Tab. 2).
**Table 2. Relation between buoyancy and TT content in He-La cells**

<table>
<thead>
<tr>
<th>Buoyancy</th>
<th>pH</th>
<th>CPM</th>
<th>%TT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TT region</td>
<td>T region</td>
</tr>
<tr>
<td>A</td>
<td>7.15</td>
<td>67.0</td>
<td>75,722</td>
</tr>
<tr>
<td>B</td>
<td>5.9</td>
<td>297.9</td>
<td>328,188</td>
</tr>
<tr>
<td>A</td>
<td>7.15</td>
<td>290.6</td>
<td>321,774</td>
</tr>
<tr>
<td>B</td>
<td>5.9</td>
<td>439.9</td>
<td>422,857</td>
</tr>
<tr>
<td>A</td>
<td>7.15</td>
<td>297.9</td>
<td>328,188</td>
</tr>
<tr>
<td>B</td>
<td>5.9</td>
<td>439.9</td>
<td>422,857</td>
</tr>
</tbody>
</table>

The values are from two experiments.

Cells were marked with thymidine-6-3H and after UV irradiation with 300 ergs/mm² were cultivated for 12 hours at the indicated pH. The TT content was estimated in the cells attached (A) to the dish and buoyant (B) respectively. Unirradiated controls were 0.003 %T at time zero and 0.002 %T (B) or 0.007 %T (A) after 12 hr cultivation at pH 5.9.

The nuclear pH also exhibits a certain variation capacity [20]. It also appears from some results that by changing extracellular pH it is possible to induce a change of the intracellular environment and thus also changes of enzymatic reactions [13].

We cannot conclude from our findings, whether the absence of excision repair in rodent cell lines is an effect of extra- or intracellular pH. Our results only suggest a possible influence of such a simple factor on repair. It is even possible that in the experimental conditions chosen by ourselves that factor became effective at another place than in the repair enzymes.

Taking into consideration the similar features of excision repair after some alkylating carcinogens and after UV irradiation [9, 11, 18], it is possible to assume that the carcinogenic effects under non-physiological acidification of extracellular environment can be potentiated.

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**References**


