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PROLIFERATIVE KINETICS OF HUMAN LYMPHOCYTES TREATED WITH CLOPROSTENOL *IN VITRO*

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Cloprostenol, a synthetic analogue of prostaglandin $F_{2\alpha}$ is widely used in for oestrus synchronization in domestic animals. Previous investigations have revealed a certain genotoxic potential of $PGF_{2\alpha}$ and cloprostenol on cultured human lymphocytes. The aim of this investigation was to evaluate the effects of cloprostenol on mitotic activity of human lymphocytes in vitro. Four experimental concentrations of cloprostenol (1, 2, 5 and 10 μg/ml) were used. The results obtained indicate that cloprostenol did not change the percentage of cells in mitosis, or the proliferative kinetics of cultured human lymphocytes.

Key words: Cloprostenol, lymphocyte, mitotic index, proliferation index

INTRODUCTION

Hormone related cancers comprise about 30% of all reported cases in developed countries (Henderson et al., 1982). Despite some evidence about the genotoxic action of hormones and their metabolic derivatives (Moldeus et al., 1983; Liehr,1990), it is also well established that hormones play an important role in carcinogenesis via epigenetic mechanisms, primarily by stimulation of cell division and proliferation (Cohen and Ellwein, 1990).

Steroid hormones can be considered as complete carcinogens capable of inducing genotoxic effects (Sawada and Ishidate, 1978; Kochhar, 1988), as well as stimulating mitotic cell division (Hsueh et al., 1975). On the other hand, the significance of nonsteroidal hormones and mediators in the process of carcinogenesis has not been thoroughly investigated.

Cloprostenol, a synthetic analogue of prostaglandin $F_{2\alpha}$, is widely used in for oestrus synchronization in sheep and cattle. Although cloprostenol expressed genotoxic effects in SCE *in vitro* (Đelić et al., 1994), the results of genotoxicity testing in cytogenetic tests *in vitro* and *in vivo* were negative (Đelić et al, 1997).

Having in mind experimental data that prostaglandin $F_{2\alpha}$ (PGF_{2a}) is involved in the development of certain cancers and that some cancers contain elevated levels of $PGF_{2\alpha}$ (Levine, 1981), we decided to explore the effects of

cloprostenol on mitotic activity of cultured human lymphocytes. Therefore, the objective of this investigation was to examine whether cloprostenol causes significant changes in mitotic activity and progression through the cell cycle of human lymphocytes in culture.

MATERIALS AND METHODS

Human peripheral blood lymphocyte cultures were prepared according to a slight modification of the protocol described by Evans and O'Riordan (1975). Heparinised whole blood samples (0.8 ml) obtained from healthy men under 35 years of age were added to vials with 9.2 ml of Parker 199 medium containing 30% of inactivated calf serum (Serva) and 0.04 mg/ml of phytohaemagglutinin (Murex). At the beginning of incubation, 5-bromo-2'-deoxyuridine (BrdUrd, Sigma Chemical Co.) was added to each culture to obtain a final concentration of 25 μM. Cultures were incubated for 72 h at $(37+0.5)^0$C.

Exactly 47 h and 30 min. after the beginning of incubation, cloprostenol (CAS No. 40665-92-7, Sinhrogal® ICN Galenika) was added to culture vials in such amounts to obtain final experimental concentrations of: 1, 2, 5 and 10 μg/ml. A negative control was prepared in ICN Galenika is an equimolar solution of all coponents of Sinhrogal®, except cloprostenol. Positive controls were N-methyl-N' nitro-N-nitrosoguanidine (MNNG) at a final concentration of 10^{-6} M, and 10^{-8} M human recombinant insulin (Inutral® HM-100, ICN).

Two hours before harvesting, colcenid (Ciba) was added to the cultures to achieve a final concentration of 0.5 μg/ml. After standard chromosome preparation, microscopic slides were stained accordilng to the FPG procedure (Perry and Wolff, 1974). Mitotic index was determined on 1000 or more cells, whereas cell cycle kinetics was estimated from the proliferation index (Tice and Ivett, 1985) scored on 200 metaphases. Proliferation index was calculated according to the formula:

$$\text{Proliferation index} = \frac{1}{100} \times (M_1 + 2M_2 + 3M_3)$$

- M_1, M_2 and M_3 refer to the percentage of metaphases in the first, second and third mitotic cycle.

Statistical analysis of the mitotic index was performed by Student's t-test, whereas proliferation index was analyzed by the X^2 test.

RESULTS

The results obtained for mitotic index in cultures of human lymphocytes treated with cloprostenol are presented in Table 1. The percentage of mitoses in cultures treated with the negative control was 4.90%. Treatment with cloprostenol did not change MI valves significantly in comparison to the negative control (range of MI from 5.16% to 6.19%). As for the positive controls, we used an agent that decreased MI values (MNNG), as well as an agent that increases MI (human recobinant insulin). The stimulative effects of insulin, as

demonstrated previously (Đelić and Soldatović, 1998), were optimal at a final insulin concentration of 10^{-8} M.

Table 1. Mitotic index of cultured human lymphocytes treated with cloprostenol

Concentration of cloprostenol	Number of cells observed	Mitotic index (%)	Percentage of control value
untreated	1142	5.08	100.00
negative control	1225	4.90	96.46
10^{-6} M MNNG	1122	3.57**	69.69
10^{-8} M insulin	1093	8.23**	162.01
1 μg/ml	1214	5.60	110.24
2 μg/ml	1008	5.16	101.57
5 μg/ml	1017	6.19	121.85
10 μg/ml	1138	5.98	117.72

** $p<0.01$ (Student's t-test)

Proliferation index is a parameter of cell cycle kinetics, that expresses the ratio of cells in the first, second and third mitotic division. In order to obtain microscopically observable differences between cells in the first, second and third mitotic divisions, we used a specific staining technique (FPG- "fluorescence plus Giemsa", Perry and Wolff, 1974).

Table 2. Proliferative kinetics in cultures of human lymphocytes treated with cloprostenol

Conc. of cloprostenol	No of cells observed	M_1		M_2		M_3		PI
		No	%	No	%	No	%	
untreated cultures	200	64	32.0	117	58.5	19	9.5	1.77
negative control	200	74	37.0	112	56.0	14	7.0	1.70
10^{-6} M MNNG	200	108	54.0	86	43.0	12	6.0	1.58**
10^{-8} M insulin	200	35	17.5	141	70.5	24	12.0	1.94**
1 μg/ml	200	65	32.5	109	54.5	16	8.0	1.66
2 μg/ml	200	75	37.5	110	55.0	15	7.5	1.70
5 μg/ml	200	66	33.0	120	60.0	14	7.0	1.74
10 μg/ml	200	72	36.0	110	55.0	18	9.0	1.73

M_1 M_2 and M_3 refer to the percentage of cells in the first, second and third mitotic cycle. respectively. PI = proliferation index

$$\text{Proliferation index} = \frac{1}{100} \times (M_1+2M_2+3M_3);\ **p<0.01;\ X^2$$

The values of proliferation indices (Table 2) clearly demonstrate that most of the cells, both in treated and control cultures, were in the second mitotic division, except after treatment with the positive control MNNG that caused

suppression of mitotic activity when most of the cells were in their first mitosis. In these experiments we used MNNG as a positive control capable of decreasing mitotic activity, and insulin as an agent that produces the opposite effect, - an increase of mitotic activity of cultured human lymphocytes.

Since proliferation index in cloprostenol-treated cultures did not departe significantly from negative control values, it can be concluded that cloprostenol did not influence cell cycle kinetics under the in vitro conditions of this investigation.

DISCUSSION

Short-term cultures of phytohaemagglutinin (PHA)-stimulated lymphocytes are widely used in cytogenetic, biochemical and immunological studies. Examination of the changes in mitotic activity and cell cycle kinetics of cultured human lymphocytes is useful for evaluation of mitogenic or cytotoxic effects of various chemical agents. Generally, increased mitotic activity underlies epigenetic mechanisms of carcinogenesis, whereas cytostatic effects point to a decreased rate of DNA replication (Đelić, 1998).

After stimulation of peripheral blood lymphocytes with PHA, the cultures soon contain different generations of cells, i. e. cells that have divided a different number of times (Morimoto et. al., 1983). The heterogeneity of cell division reflects either a difference in cell cycle duration, or a difference in the times when the cells start blastogenesis in response to PHA (Sinha et al., 1984).

Perry and Wolff (1974) developed a sensitive method for differential staining of sister chromatids (known as FPG - Fluorescence plus Giemsa), capable of visualizing metaphase spreads isolated from cells in the first, second and third mitotic divisions. The same technique is also used as a very sensitive test for detection of DNA damage following exposure to chemical genotoxins.

In this study, we analyzed the possible effects of cloprostenol (a synthetic analogue of prostaglandin F_{2a}) on proliferative kinetics of human lymphocytes *in vitro*. Our previous studies showed that cloprostenol elevates SCE per cell frequency in cultured human lymphocytes (Đelić et al., 1994). Therefore, cloprostenol may contribute to the formation of genetic lesions involved in the process of tumor initiation. On the other hand, analysis of proliferative kinetics should detect a possible role of cloprostenol in tumor promotion (epigenetic mechanism of carcinogenesis).

The results obtained clearly indicate that cloprostenol did not influence mitotic activity, or change the values of proliferation index as a parameter of cell cycle kinetics. It should be mentioned, however, that prostaglandin $F_{2\alpha}$ can cause chromatin decondensation of small and large luteal cels, without increase in nuclear volume (Chegini et al., 1991). Changes in nuclear volume and chromatin condensation are generally considered to reflect altered gene expression in eukaryotic cells. Nuclear volume and chromatin decondensation can increase during the cell cycle as a result of increased nucleic acid and protein synthesis. On the other hand, Sawyer et al. (1990) demonstrated that $PGF_{2\alpha}$ is involved in nuclear changes indicative of apoptosis in luteal endothelial

cells. It seems conceivable that the failure of cloprostenol to induce changes in proliferative kinetics of human lymphocytes may be explaind by different reactivity of chromatin in lymphocytes after the action of cloprostenol.

The complexity of signal transduction pathways in response to $PGF_{2\alpha}$ is well documented by numerous experimental data from various laboratories. Thus, it has been shown that after $PGF_{2\alpha}$ binds to specific membrane receptors, the following intracellular events may occur (depending on cell type or developmental stage): elevated cytosolic free Ca^{2+} concentrations (Rodway et al., 1991), activation of inositol-phospholipid signal transduction pathway (Nakada et al., 1990), stimulation of phospholipase C (Hakeda et al., 1991) and increase of membrane associated protein kinase C activity accompanied by modulation of cAMP response (Wada et al., 1991).

At present, possible subcellular events in human lymphocytes responsible for the genotoxic effects of $PGF_{2\alpha}$ and its analogue cloprostenol (Das et al., 1989; Đelić et al., 1994) have not been revealed. The attempt to examine whether some of the above mentioned molecular mechanisms might be (in) directly involved in the processes of mutagenesis and/or modulation of DNA synthesis accompanied by changes in mitotic and proliferative activity requires further investigations by various techniques currently used in molecular biology and cytobiochemistry.

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KINETIKA PROLIFERACIJE HUMANIH MIFOCITA TRETIRANIH KLOPROSTENOLOM *IN VITRO*

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SADRŽAJ

Kloprostenol je sintetički analog prostaglandina $F_{2\alpha}$ koji se upotrebljava za sinhronizaciju estrusa domaćih životinja. Prethodna ispitivanja otkrila su izvestan genotoksični potencijal $PGF_{2\alpha}$ i kloprostenola na humanim limfocitima u kulturi. Cilj ovog rada bila su istraživanja mogućih efekata kloprostenola na mitotsku aktivnost humanih limfocita *in vitro*. Ispitane su četiri eksperimentalne koncentracije (1, 2, 5 i 10 mg/ml). Dobijeni rezultati ukazuju da kloprostenol ne menja značajno procenat ćelija u mitozi i ne utiče na kinetiku proliferacije humanih limfocita u kulturi.