SHORT COMMUNICATION

Reduction of glutathione content by 12-O-tetradecanoylphorbol-13-acetate in confluent, but not in sparse cultures of human diploid fibroblasts

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Treatment of confluent cultures of human diploid fibroblasts with 12-O-tetradecanoylphorbol-13-acetate (TPA) (10^-7 M) resulted in a 70% reduction of the glutathione (GSH) content, compared with untreated controls. The effect, which was dose-dependent, was observed 8 h after the beginning of the treatment and could be followed for up to 72 h. On the other hand, GSH reduction was specific for confluent cultures, as the level of glutathione remained unchanged by TPA treatment of sparse cultures. The addition of immobilized plasma membrane proteins to sparsely seeded cells has been shown previously to induce cellular reactions which are characteristic for confluent cultures. It was shown that TPA treatment of sparse cultures grown in the presence of immobilized plasma membrane proteins also resulted in a 70% reduction of glutathione content. These data agree with the postulated involvement of redox reactions in tumor promotion, and point to a central role of cell-cell contacts in the regulation of biochemical events which are critical in tumorigenesis.

Under physiological conditions the concentration of free radicals produced by the cells from molecular oxygen is maintained through enzymatic and non-enzymatic reactions at an acceptable low level by an elaborate defence system consisting of the anti-oxidant enzymes sodium oxide dismutase, catalase and glutathione peroxidase, and low mol. wt non-protein sulfhydryls, i.e. glutathione (GSH), cysteine and cysteinyl-glycine. Vitamin E and C, β-carotene and urate provide additional anti-oxidants (1-4). In this study, the effect of 12-O-tetradecanoylphorbol-13-acetate (TPA), a known inducer of unphysiologically high amounts of oxygen radicals in a number of cell systems (5-9), on GSH content of human diploid fibroblasts in relation to the actual cell density was investigated.

The cells used in this study and cell culture conditions are described elsewhere (10). Solubilization of plasma membrane proteins and immobilization of solubilized plasma membrane proteins to derivatized silica beads was done according to (11). Briefly, plasma membrane proteins were solubilized by in situ incubation of washed cell monolayers with 4 mM CHAPS [3-(cholamidopropyl)-dimethylammonio(-1,1-propanesulfonate)] in phosphate-buffered saline (PBS) for 30 min at 4°C. The solution was centrifuged to remove non-solubilized cellular components, and the solubilized proteins were coupled covalently to 10 μm silica beads (Lichrospher Diol Si 500 SA, Merck, Darmstadt), which had been derivatized with 3-isothiocyanatopropyltriethoxysilane. Covalent coupling occurs by the reaction of the SCN group of the isothiocyanatopropylsilica with the amino groups of the proteins. After different washing steps with PBS, Dulbecco’s modified Eagle’s medium (DMEM) and DMEM/10% fetal calf serum (FCS) the beads were added to the test cells. Controls received beads reacted with DMEM alone or with bovine serum albumin (BSA). Control beads were without effect on the growth behaviour of the cells. Determination of total glutathione content was performed according to (12).

First experiments were done to study temporal changes of the GSH/oxidized glutathione (GSGS) content after TPA (10^-7 M) treatment of confluent cultures of human diploid fibroblasts. As shown in Figure 1, a significant decrease in the total GSH content was observed 8 h after the addition of TPA, resulting in a 70% reduction after 24 h. These low levels of GSH were still observed after 72 h, raising the question of whether this effect resulted from the continuous presence of TPA. A 50% reduction in total GSH content was found 24 h after a 3 h treatment with TPA (Figure 2), indicating a long lasting effect of TPA on the GSH levels, i.e. on its reconstituting enzymes. This long duration of the effect of TPA indicates the generation of free radicals from molecular oxygen and subsequent consumption of GSH to detoxify these compounds. A decline in total intracellular GSH as a consequence of the action of xenobiotics or ‘oxidative stress’ has also been shown for other cell systems.

The reduction of the GSH content has been found to be strongly dependent upon the concentration of TPA added to the cells (Figure 3). Interestingly, 10^-5 M TPA showed no further decrease compared with 10^-6 M. Since in human fibroblasts different effects of these two concentrations of TPA have been found with respect to proliferation, although TPA even at a concentration of 10^-5 M is not toxic, as shown in various proliferation assays (13) (data not shown), it is likely that the failing effect is to be explained by free radical related circumstances. It is possible that the generation of oxygen radicals has its peak at 10^-5 M with no further increase at higher concentrations.

Previous studies have shown that TPA exerted opposite effects on the proliferation of human fibroblasts with respect to cell density (14). While the growth of sparsely seeded cells was inhibited by TPA, confluent cultures showed an increased proliferation rate. In addition, cell cycle-dependent variations of the glutathione levels have been reported (15-17). As shown in Figure 4, no differences in GSH content between untreated sparse and confluent cultures of human fibroblasts were observed. In contrast, in dense cultures a significant decrease in GSH content was found after TPA treatment (24 h, 10^-7 M). Again, no change occurred in sparse cultures. Since it can be expected that TPA induces the same cellular reactions on a per cell basis in both cell types to the same degree, some constitutive differences such as different enzyme activities may be responsible for the observed effects. Whether the different effects of TPA on dense and sparse cultures of human fibroblasts reflect cell cycle-specific stages, remains to be elucidated. It should, however, be mentioned that treatment of dense cultures with TPA results in...
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Fig. 1. TPA treatment of human diploid fibroblasts leads to time-dependent depletion of intracellular total GSH content. Confluent cultures (1 × 10^5/cm^2) were treated with 10^{-7} M TPA or, as controls, with dimethylsulphoxide. After the culture periods indicated, total GSH content was determined in triplicate samples. Values are given as % of controls ± SD.

Fig. 2. Short-term treatment (3 h) with TPA (10^{-7} M) of confluent cultures of human diploid fibroblasts leads to a 50% reduction of total GSH content after a 24 h culture period. Confluent cultures were treated for 3 h with TPA and cultured subsequently for 24 h, after which total GSH content was determined. Values of three independent experiments are expressed as nmol GSH equivalents ± SD/mg protein.

Fig. 3. The degree of depletion of total glutathione is dependent upon the concentration of TPA added to confluent cultures of human diploid fibroblasts. Confluent cultures of human fibroblasts were treated for 24 h with the indicated TPA concentrations, and total GSH content was determined. Values given as in Figure 2.

Fig. 4. The effect of TPA on total GSH content depends on the actual cell density. GSH content was determined in confluent and sparse cultures of human fibroblasts with or without TPA treatment for 24 h. In addition, sparse cultures were grown in the presence of silica beads with immobilized plasma membrane proteins in the presence or absence (controls) of TPA. Values are given as in Figure 2.

Fig. 5. The degree of depletion of total glutathione is dependent upon the concentration of TPA added to confluent cultures of human diploid fibroblasts. Confluent cultures of human fibroblasts were treated for 24 h with the indicated TPA concentrations, and total GSH content was determined. Values given as in Figure 2.

A proliferative burst (14), while sparsely seeded cells are growth inhibited in the presence of TPA.

A number of experiments give evidence for the involvement of plasma membrane glycoproteins in the contact-dependent regulation of growth and differentiation (18–22). In human fibroblasts it has been shown that the addition of fixed fibroblasts, isolated plasma membranes or immobilized plasma membrane glycoproteins to sparsely seeded cells resulted in a strong inhibition of growth and an enhanced synthesis of collagen III (10,11,23). As both cellular reactions are characteristic for confluent cultures, this indicates that these systems are able to imitate, in sparsely seeded cells the situation occurring in dense cultures. The addition of immobilized plasma membrane proteins to sparsely seeded cells, grown in the presence of TPA, reduced the GSH content to almost the same value as found in confluent, TPA-treated cultures (Figure 4). The addition of immobilized plasma membrane proteins itself was without effect on the GSH content. Although we have no experimental proof, it is suggested that the observed effects of TPA are related to the generation of oxygen radicals (24–31), and that cell–cell contacts directly influence the anti-oxidant machinery by changing the biochemical state of the cells. Imitation of cell–cell contacts has been shown to not only influence the proliferative behaviour, but also the expression of more mature functions of cells (11,20). Since
confluent cultures of fibroblasts represent a more physiological in vivo equivalent than do sparsely seed cells (as shown by the following cellular reactions, which are common to both the fibroblasts in confluent cultures and in vivo: stop of proliferation; enhanced synthesis of collagen I and III; enhanced synthesis of fibronectin) it is suggested that in an in vivo situation, i.e. under non-proliferative conditions, TPA may exert more severe injuries than under proliferative circumstances.

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References