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PHORBOL ESTER-INDUCED REORGANIZATION OF THE CYTOSKELETON IN HUMAN FIBROBLASTS DURING AGEING IN VITRO

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Phorbol esters induce drastic morphological alterations in cells of different origin by altering the conformation and the interrelationship of the elements of the cytoskeletal system. Treatment of early passage (young) and late passage (senescent) human fibroblasts MRC-5 with phorbol-12-myristate-13-acetate (PMA) results in the rearrangement of actin and tubulin filaments. PMA brings about the disorientation and diffusion of the heavily criss-crossed network of actin and microtubulin fibres characteristic of senescent cells suggesting thereby an increased sensitivity of senescent cells to phorbol esters. Since phorbol esters are known to be specific activators of protein kinase C (PKC), the PMA-induced modulation of the cytoskeleton patterns in ageing fibroblasts provides further support for the view that the effectiveness of the signalling mechanisms is retained during cellular ageing.

One of the major cellular effects of phorbol esters is the reorganization of the cytoskeleton as a result of induced alterations of the interrelationship between the microtubular system and the actin cortex. Changes in the cytoskeleton elements, such as actin [1-4], calspectin [5], vimentin [6] and the microtubules [7] have been reported for human blood cells, fibroblasts and rodent cells treated with phorbol esters. It has also been shown that phorbol esters affect the morphology, contractility, adhesion and locomotion of cells by inducing the reorganization of the actin filaments and the microtubules [7,8]. Furthermore, treatment of fibroblasts with phorbol esters induces the disruption of stress fibres and the loss of focal adhesions, resulting in drastic morphological changes and a loss of substrate dependence [9,10] .

Since changes in the cytoskeletal pattern are a major characteristic of cellular ageing [11,12], the use of phorbol esters to modulate cytoskeleton in ageing cells can facilitate the determination of the extent of its involvement in age-related functional changes, such as altered cellular responsiveness to external stimuli. For example, a decreased response to growth factors and an increased sensitivity to toxic factors are phenomena widely observed during ageing [13]. In the case of phorbol esters, the phorbol myristate acetate (PMA)-induced DNA synthesis was shown to be markedly lower in blood lymphocytes from old subjects [14]. The PMA-induced activation of NADPH-oxidase is increased in neutrophils of aged human donors [15]. Furthermore, phorbol esters have an inhibitory effect on the glucagon-induced adenylate desensitization only in hepatocytes of aged rats and not in foetal cells [16]. There is also a

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report of an age-related decline of the effect of PMA on actin polymerization in human polymorphonuclear leukocytes, as observed by estimation of the amount of monomeric actin in cell extracts [1].

Although there is some information available on the effects of phorbol esters on cytoskeleton organization of immortal and transformed mammalian cells [17,18], nothing is known about this aspect of normal diploid cells undergoing ageing in culture. Therefore, we have tested the effects of PMA on the cytoskeletal organization of serially passaged young and old human fibroblasts in culture, by fluorescence microscopic observations. Our results show that PMA mobilizes the cellular cytoskeleton from a highly polymerized state in senescent cells to a diffused organization. These results also suggest that old cells may be more sensitive to the effects of phorbol esters and that the pathways of PMA-induced signal transduction are largely unaltered during cellular ageing.

MATERIALS AND METHODS

Cell culture. Normal human embryonic diploid lung fibroblasts, MRC-5, were cultured routinely in Dulbeco's modified Eagle's minimum essential medium (DMEM, Biochrom, FRG) containing 10% foetal calf serum and antibiotics at 37°C in an atmosphere of 5% CO_2, as described before [19]. The lifespan of MRC-5 cells *in vitro* was estimated by calculating the cumulative population doubling level (CPDL) attained on 1:2 serial passaging. In the present series of experiments, MRC-5 cells reached CPDL 49, which is here considered as 100% lifespan. Following the terminology of the Hayflick system, cultures were considered to be in Phase II until slowing-down of their growth during the last few passages (Phase III; senescence) when they leave the cycle irreversibly.

At various periods of their lifespan, about 6 x10^4 cells were seeded in multi-chamber slides (chamber area 2 cm^2; Teknunc) in normal medium and cells were allowed to grow for 7 days. After this period, the culture medium was replaced by fresh medium containing a lower concentration of serum (0.2%) for 48 hr. This was done in order to synchronize the culture in G1 phase of the cell cycle (more than 99% of the cells were arrested in G1 as checked routinely by [H^3]thymidine autoradiography). Quiescent cells were then treated with various doses (1-100 ng/ml) of PMA (4-β-phorbol-12-β-myristate-13-α-acetate; Sigma) dissolved in DMSO, by its addition to fresh low serum culture medium. After different durations of PMA treatment, cells were processed for actin and tubulin staining as follows.

Immunostaining for microtubules. After removal of the culture medium, cells were washed three times in Hank's buffer and then fixed in cold (-20°C) methanol for 30 min. Cultures were then washed twice with distilled water. The microtubule pattern of the cells was analysed by indirect immunofluorescence method. Cells were first treated with mouse monoclonal anti-tubulin antibody (Amersham) at a 1:500 dilution and incubated at 37°C for 60 min. Free antibodies were removed by washing the cells three times in distilled water. A second antibody (FITC-conjugated IgG; Amersham) was then added in order to make anti-tubulin antibody fluorescent. After a further 60 min incubation at 37°C, cells were washed three times with distilled water, air-dried and covered with a glass coverslip using galvatol adhesive.

Phalloidin staining of actin. Cells in chamber-slides were washed three times with Hank's buffer and were fixed at room temperature first in 3.5% formaldehyde for 30 min and then in acetone for 15 min. After washing two times with distilled water, cells were incubated with FITC-labelled phalloidin (Sigma) for 45 min, followed by five washings in distilled water. A Zeiss Axiophot fluorescence microscope was used to observe the fluorescent pattern of the cytoskeletal elements of the cells.

RESULTS

Treatment of normal human fibroblasts with PMA causes drastic reorganization of the cytoskeletal network by inducing disassembly of the actin and tubulin filaments in both young and old fibroblasts. Fig. 1 shows the effect of PMA on the pattern of actin filaments in young and old MRC-5 cells. Untreated young fibroblasts in Phase II (less than 50% lifespan completed) have partly organized actin cytoskeletal pattern with only a few fibrillar microfilaments present (Fig. 1a). Actin is less extensively polymerized in Phase II cells of low population doublings than in Phase III cells of higher population doublings. Senescent MRC-5 fibroblasts (more than 98% lifespan completed) exhibit a heavy network of nonmotile and overcrossing cytoskeletal fibre-like formations of actin filaments (Fig. 1c).

In response to PMA (100 ng/ml; 24 hr), actin patterns of young MRC-5 cells become disoriented and diffused (Fig. 1b). In comparison, senescent cells treated with PMA (100 ng/ml; 24 hr) lose their rod-like actin fibres and the depolymerized actin appears in bundle-formations, concentrated mainly around the nucleus and the cell membrane (Fig. 1d). Equivalent concentrations of PMA begin to induce reorganization of the actin network already after an hour of treatment, irrespective of the CPDL status of the cells *(pictures not shown)*. It should be noted that at similar doses of PMA treatment, the extent of diffusion and disassembly of the fibre-like structures of highly polymerized actin microfilaments in Phase III senescent

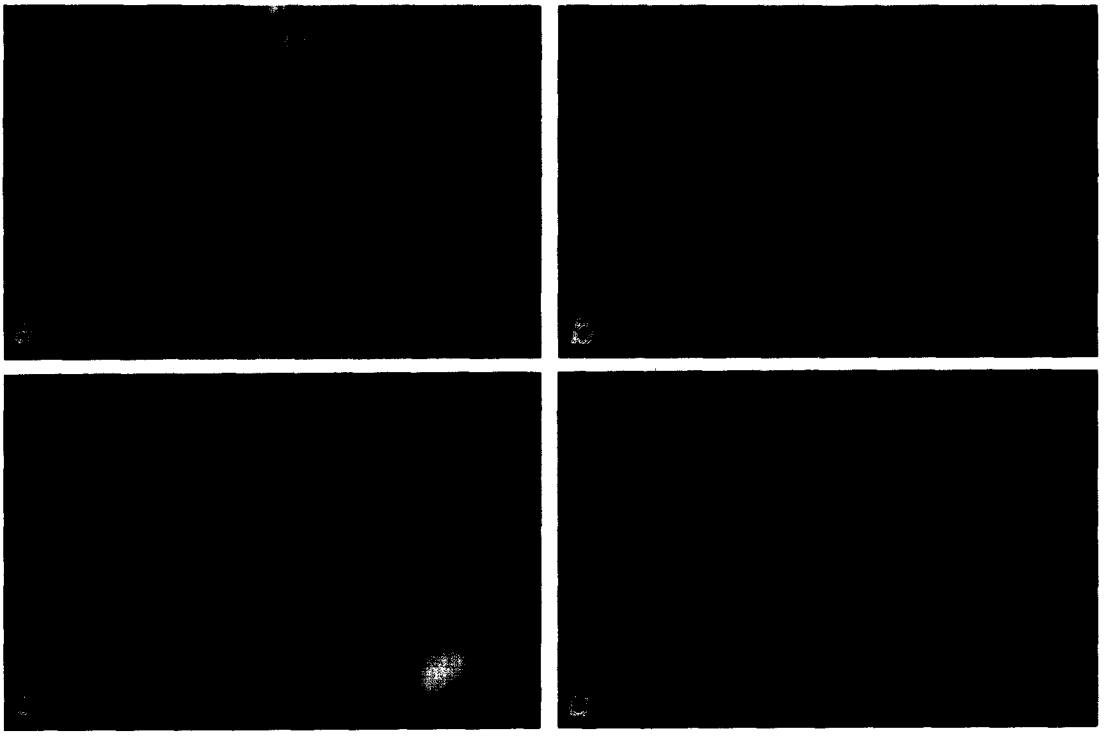

Fig. 1. Effect of PMA (100 ng/ml for 24 hr) on the phalloidin-stained fluorescent pattern of actin filaments in young and old MRC-5 cells. (a, b): Phase II young cells of less than 50% lifespan completed without and with PMA, respectively. (c, d): Phase III senescent cells of more than 98% lifespan completed without and with PMA, respectively.

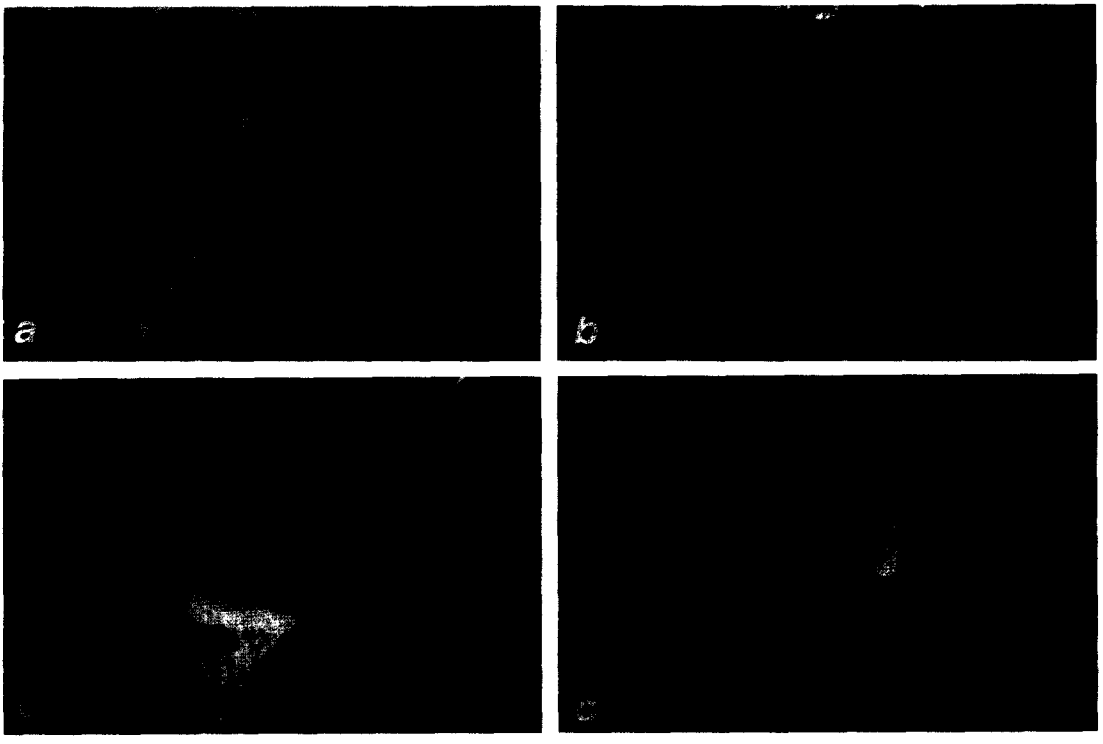

Fig. 2. Effect of PMA (100 ng/ml for 24 hr) on the immunofluorescent staining pattern of microtubules in young and old MRC-5 cells with and without PMA treatment. (a, b): Phase II young cells of less than 50% lifespan completed without and with PMA, respectively. (c, d): Phase III senescent cells of more than 98% lifespan completed without and with PMA, respectively.

cells is higher than in Phase II cells, indicating that the sensitivity of old cells to PMA is increased during ageing.

PMA treatment also induces disassembly and mobilization of microtubules. Fig. 2 shows the microtubular patterns of young and old MRC-5 cells with and without PMA-treatment. The tubulin network is more orderly in young cells (Fig. 2a) than in aged cells (Fig. 2c) which exhibit a heavily criss-crossed pattern. The microtubular patterns become diffuse in response to PMA (100 ng/ml; 24 hr) both in young and senescent cells (Fig. 2 b, d). The PMA-induced diffuse pattern of tubulin appears in Phase II young cells in dot-like bundles in the cytoplasm and mostly around the nucleus (Fig. 2b). In the case of Phase III senescent cells, the heavily criss-crossed microtubules become severely disassembled and disoriented after treatment with PMA (Fig. 2d). Comparing the extents of microtubular disorganization induced by the same doses of PMA in young and old cells, implies that senescent cells may be more sensitive to the effects of PMA.

DISCUSSION

The PMA-induced cytoskeletal alterations in relation to cellular ageing provide new data regarding the age-related changes of cellular responsiveness, an aspect of great importance

during ageing [20,21]. Although the effects of phorbol esters on the conformation and the interrelationships of the cytoskeletal elements have been reported for human blood cells [1] and immortal and transformed human and rodent cells [17,18], very little is known in the context of cellular ageing in culture. The present results about the PMA-induced reorganization of the actin and tubulin cytoskeletal patterns during cellular ageing imply an age-related increase of cellular sensitivity to PMA. These conclusions are derived from the fact that the same concentrations of PMA, which induce the reorganization of the cytoskeleton of young human fibroblasts, also alter the heavy cytoskeletal network of senescent fibroblasts. PMA induces the mobilization of the cellular cytoskeletal state in senescent cells by modulating the excessively polymerized actin and tubulin present in these cells and results in a similar diffuse pattern of cytoskeleton in senescent cells as in young cells. The extent of disassembly of the cytoskeleton patterns is considered to be greater in ageing cells, since the levels of complexity and immobilization of the cytoskeleton is higher in these cells. These results indicate a greater effect of the phorbol esters on ageing cells and, consequently suggest an increase of sensitivity to the effect of PMA on cytoskeleton during ageing.

Furthermore, these results not only support the generally accepted view of the increased sensitivity of cells to tumour promoters and other toxic factors, they also yield information about the effectiveness of the signal-transduction pathways during cellular ageing. It is well known that the effect of phorbol esters on cell gene expression is mediated by the activation of PKC [22]. In addition, PKC is known to affect the conformation of actin, actin-binding proteins and their interactions [23]. PKC also plays a role in the regulation of tubulin-polymerization and its interaction with actin [24]. Therefore, the PMA-induced reorganization of the cytoskeleton in senescent cells indicates a functionally intact pathway of signalling during cellular ageing. The efficiency of signal transduction pathways during ageing has also been previously indicated by the retention by senescent cells of their ability to respond to receptor-binding stimuli by expressing specific genes [25,26], some of which are also PMA-inducible primary-response genes [27].

The action of PMA on the cytoskeleton is one of the primary effects of this phorbol ester and is a part of the cascade of changes evoked in the signalling pathways and resulting in the modulation of gene expression. In addition, phorbol esters have been shown to have several differential effects on cells, which are dependent on the origin of the cells [28-30] and their cell-cycle status [31,32], suggesting that there may be differential cellular effects of phorbol esters during cellular ageing as well. Indeed, there are reports showing that phorbol esters may or may not increase the proliferative capacity of human fibroblasts and Syrian hamster cell cultures depending upon the age of the cells at the time of initiation of the treatment [33,34]. Similarly, the reduced effect of phorbol esters on the DNA-synthesis of T-cells from old humans [14] has been explained by a loss of PMA-susceptible subpopulations rather than gradual loss of the responsiveness of the whole population of cells [35].

Further studies will clarify the nature and extent of differential effects of phorbol esters on different cell types and under different conditions, including cell-cycle, ageing and immortalization. Studying the age-related effects of PMA on gene expression will be the next logical step in order to determine age-dependent alterations of cellular responsiveness.

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REFERENCES

1. Rao, K. M. K. (1986) *J. Gerontol.* **41,** 561-566.
2. Nabika, T., Chaldakov, G. N., Nara, Y., Endo, J., and Yamori, Y. (1988) *Exp. Cell Res.* **178,** 358-368.
3. Phatak, P. D., Packman, C. H., and Lichtman, M. A. (1988) *J. Immunol.* **141,** 2929-2934.
4. Keller, H. U., Nggli, V., and Zimmermann, A. (1989) *J. Cell Sci.* **93,** 457-465.
5. Sobue, K., Fujio, Y., and Kanda, K. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 482-486.
6. Giese, G., Kubbies, M., and Traub, P. (1990) *Exp. Cell Res.* **190,** 179-184.
7. Lyass, L. A., Bershadsky, A.D., Vasilliev, J. M., and Gelfand, I. M. (1988) *Proc. Natl. Acad. USA* **85,** 9538-9541.
8. Sastrodihardjo, S., Sasaki, Y., Shiba, Y., and Kanno, Y. (1987) *J. Cell. Physiol.* **132,** 49-56.
9. Danowski, B. A., and Harris, A. K. (1988) *Exp. Cell Res.* **177,** 47-59.
10. Meigs, J. B., and Wang, Y. -L. (1986) *J. Cell Biol.* **102,** 1430-
11. Rao, K. M. K., and Cohen, H. J. (1990) *Exp. Gerontol.* **24,** 7-22.
12. Raes, M. (1991) *Mutat. Res.* **256,** in press
13. Rattan, S. I. S., and Derventzi, A. (1991) *BioEssays* **13,** 601-606.
14. Nagel, J. E., Chrest, F. J., and Alder, W. H. (1982) *Clin. Exp. Immunol.* **49,** 217-224.
15. Scott, R. B., Wood, H. E., Matin, S., Kukreja, R. C., and Hess, M. L. (1990) *Exp. Gerontol.* **25,** 523-532.
16. Hermsdorf, T., Dettmer, D., and Hofmann, E., (1989) *Biomed. Biochim. Acta* **48,** 255-260.
17. Shiba, Y., Sasaki, Y., and Kanno, Y. (1988) *Exp. Cell Res.* **178,** 233-241.
18. Tint, I. S., Bershadsky, A. D., Gelfand, I. M., and Vasiliev, J. M. (1991) *Proc. Natl. Acad. USA* **88,** 6318-6322.
19. Cavallius, J., Rattan, S. I. S., and Clark, B. F. C. (1986) *Exp. Gerontol.* **21,** 149-157.
20. Cristofalo, V. J., Phillips, P. D., Sorger, T., and Gehard, G. (1989) *J. Gerontol.* **44,** B55-B62.
21. Derventzi, A., and Rattan, S. I. S. (1991) *Mutat. Res.* **256,** in press
22. Nishizuka, Y. (1984) *Nature* **308,** 693-697.
23. Bähler, M., and Greencard, P. (1987) *Nature* **326,** 704-707.
24. Sattilaro, R. F. (1986) *Biochem.* **25,** 2002-2009.
25. Rittling, S. R., Brooks, K. M., Cristofalo, V. J., and Baserga, R. (1986) *Proc. Natl. Acad. USA* **83,** 3316-3320.
26. Shigeoka, H., and Yang, H. C. (1990) *Mech. Ageing Dev.* **55,** 49-59.
27. Herschman, H. R. (1991) *Annu. Rev. Biochem.* **60,** 281-319.
28. Corsini, E., Restani, P., Galli, L. C., and Marinovich, M. (1990) *Exp. Cell. Res.* **191,** 129-132.
29. Brooks, G., Birch, M., and Hart, I. R. (1990) *Pigment Cell Res.* **3,** 98-100.
30. Fridman, R., Lacal, J. C., Resich, R., Bonfil, D. R., Ahn, C.-H. (1990) *J. Cell. Physiol.* **142,** 55-60.
31. Böhmer, R. M., (1988) *Growth Factors* **1,** 85-90.
32. Tsonis, P. A., and Goetinck, P. F. (1990) *Exp. Cell Res.* **190,** 247-253.
33. Diamond, L., O'Brien, S., Donalson, C., Shimizu, Y. (1974) *Int. J. Cancer* **13,** 721-730.
34. Ueo, H., Nakano, S., Ts`o, P. O. P., and Bruce, S. A. (1990) *J. Cell. Physiol.* **142,** 505-513.
35. Rivas, A. J., Kondrack, E., Machado, I., and Bianco, N. E. (1985) *Gerontol.* **31,** 39-45.