Hyperphosphorylation of Cytokeratins by Okadaic Acid Class Tumor Promoters in Primary Human Keratinocytes

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ABSTRACT

Okadaic acid, dinophysistoxin-1 (35-methylotokadacid acid), and calyculin A are potent tumor promoters on mouse skin (H. Fujiki, M. Suganuma, S. Nishiwaki, S. Yoshizawa, J. Yatsunami, R. Matsushima, H. Furuya, S. Okabe, S. Matsusaga, and T. Sugimura. In: R. D’Amato, T. J. Slaga, W. Farland, and C. Henry (eds.), Relevance of Animal Studies to the Evaluation of Human Cancer Risk, pp. 337–350. New York: John Wiley and Sons, Inc., 1992). These tumor promoters, which are also inhibitors of protein phosphatases 1 and 2A, induced hyperphosphorylation of M, 60,000, M, 58,000, M, 56,000, M, 52,000, M, 42,000, and M, 27,000 proteins in PHK 16-1 cells, human keratinocytes immortalized by human papillomavirus type 16. Except for the M, 27,000 protein, these hyperphosphorylated proteins were identified to be cytokeratin peptides (CK) CK 5, CK 6, CK 7, CK 16, and CK 19, by anti-cytokeratin antibodies. CK 5 and CK 6 were more strongly phosphorylated than CK 16 and CK 19. The in vitro hyperphosphorylation of these cytokeratins was also found by incubation with an enzyme fraction containing a mixture of protein phosphatase 2A and protein kinases isolated from mouse brain and various concentrations of dinophysistoxin-1. Indirect immunofluorescence microscopy with anti-cytokeratin antibodies revealed that the hyperphosphorylated cytokeratins had retracted to the perinuclear area. The hyperphosphorylated M, 27,000 protein was identified as a heat shock protein, HSP27. Hyperphosphorylation of HSP27 and intermediate filaments, such as cytokeratins, is one of the early biochemical changes, or pleiotropic effects, in cells induced by the okadaic acid class of tumor promoters.

INTRODUCTION

Okadaic acid, dinophysistoxin-1 (35-methylotokadacid acid), and calyculin A are potent tumor promoters of the okadaic acid class on mouse skin initiated with DMBA1 (1). They bind to the catalytic subunits of protein phosphatases 1 and 2A and inhibit their activities, resulting in the increase of phosphoproteins, i.e., the hyperphosphorylation of proteins (2, 3). This new mechanism of tumor promotion was named the okadaic acid pathway (1). In primary human fibroblasts treated with okadaic acid and dinophysistoxin-1, we have recently demonstrated that vimentin, one of the intermediate filaments, is a target protein for hyperphosphorylation (4). Therefore, we think that various types of intermediate filament proteins are generally hyperphosphorylated in various cells by treatment with okadaic acid class tumor promoters. Okadaic acid, dinophysistoxin-1, and calyculin A induced hyperphosphorylation of proteins other than vimentin similarly in PHK 16-1 cells, human keratinocytes immortalized by human papillomavirus type 16 (5). This article reports that these hyperphosphorylated proteins are cytokeratins.

Cytokeratin is the group name for the intermediate filaments present in epithelial cells. Expression of cytokeratins in the epidermis varies depending on cell types, stages of differentiation, the cellular growth environment, and skin diseases (6). Although the function of individual cytokeratins in keratinocytes is not well understood, Epstein (7) recently reported that cytokeratin function is related to mechanical stability of the cells. In this experiment, we characterized the hyperphosphorylated cytokeratins in PHK 16-1 cells through immunoprecipitation and Western blot analysis. The significance of the hyperphosphorylation of cytokeratins was discussed in accordance with their functional alteration and with the changes in intermediate filament networks that are associated with tumor promotion.

MATERIALS AND METHODS

Chemicals and Cells. Okadaic acid and dinophysistoxin-1 were isolated from the black sponge, Halichondria okadai (8). Calyculin A was isolated from a marine sponge, Discoderma calyx (9). PHK 16-1 cells were kindly provided by Dr. Shigeru Yasumoto at the Kanagawa Cancer Center Research Institute, Yokohama, Japan (10). Fetal calf serum and MCDB 152 medium were purchased from Flow Laboratories, North Ryde, New South Wales, Australia, and Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan, respectively. 32P1 was obtained from Amersham, Buckinghamshire, United Kingdom.

Antibodies. Antibody HK6 against cytokeratin peptide CK 6 was prepared by S. H. Y as described previously (11). Antibodies AE-1, AE-2, AE-3, AE-8, RCK 105, K8.13, and 19 Ks were purchased from ICN Biomedicals, Inc., Lisle, IL. Antibodies PKK-1 and PKK-2 were obtained from Labsystems, Helsinki, Finland. MAK-6 was purchased from Triton Bioscience, Inc., Alameda, CA. Anti-M, 27,000 heat shock protein antibody SPA-800 was purchased from Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada.

Hyperphosphorylation of Proteins in PHK 16-1 Cells. PHK 16-1 cells (1 x 10^6) were cultured in a phosphate-deficient MCDB medium for 14 h. 32P1 was added to the medium at a concentration of 1.85 MBq/ml and incubated for 3 h. PHK 16-1 cells were further treated for 2 h with 100 nM of an okadaic acid class tumor promoter dissolved in dimethyl sulfoxide. Cells were solubilized in lysis buffer consisting of 50 mM 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid (pH 7.4), 250 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 50 μg/ml of aprotinin, followed by centrifugation (12,000 rpm × 10 min). The soluble fraction was directly dissolved in electrophoresis buffer and applied to one-dimensional SDS-PAGE (12), followed by autoradiography.

Immunoprecipitation of Hyperphosphorylated Proteins. Hyperphosphorylated proteins present in the soluble fraction of the cell lysates were immunoprecipitated with the anti-cytokeratin antibody K8.13 and anti-M, 27,000 heat shock protein antibody SPA-800 as described previously (4). Both immunoprecipitates were subjected to one-dimensional SDS-PAGE and followed by autoradiography.

Western Blot Analysis of Hyperphosphorylated Cytokeratins. PHK 16-1 cells (1 x 10^6) labeled with 32P1, were treated with 100 nM dinophysistoxin-1 for 2 h. The soluble fraction of the cell lysates was directly dissolved in electrophoresis buffer and subjected to one-dimensional SDS-PAGE. The proteins which were transferred to a nitrocellulose membrane were further reacted with each of various anti-cytokeratin antibodies, as indicated in Table 1, and later stained with the immunoperoxidase method using the avidin-biotin complex described previously (4).

Two-dimensional Gel Electrophoresis of Cytokeratins. After labeling with 32P1, PHK 16-1 cells (1 x 10^6) were treated with either 100 nM dinophysistoxin-1 or 100 nM calyculin A and proteins of identical molecular weights to the newly synthesized labeled proteins were isolated from the cells as described previously (5).

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; CK, cytokeratin peptide; HSP27, M, 27,000 heat shock protein; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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Fig. 1. Hyperphosphorylation of proteins in PHK 16-1 cells treated with okadaic acid class tumor promoters. After labeling with $^{32}$P, cells (1 x $10^6$ cells) were incubated for 2 h with dimethyl sulfoxide as vehicle (Lane 1), 100 nM okadaic acid (Lane 2), 100 nM dinophysistoxin-1 (Lane 3), and 100 nM calyculin A (Lane 4). kDa, kilodaltons.

Indirect Immunofluorescence Microscopy. PHK 16-1 cells (5 x $10^4$/chamber), plated on 4-chamber slides, were treated with 100 nM dinophysistoxin-1 for 15 min. After fixation of the cells with methanol, the slides were treated with the anti-cytokeratin antibody AE-3 for 2 h and further incubated with secondary antibody conjugated with fluorescein for 1 h. The slides were examined microscopically.

RESULTS

Hyperphosphorylation of Proteins in PHK 16-1 Cells. One-dimensional SDS-PAGE of the soluble fraction of the lysates revealed that okadaic acid, dinophysistoxin-1, and calyculin A induced hyperphosphorylation of proteins with molecular weights of 60,000, 58,000, 52,000, 42,000, and 27,000 in PHK 16-1 cells (Fig. 1). The insoluble fraction of the lysis buffer also contained the hyperphosphorylated proteins, but the M, 27,000 phosphoprotein was mainly found in the soluble fraction. Moreover, the former four proteins are phosphorylated by only the okadaic acid class of tumor promoters and the M, 27,000 protein was phosphorylated by okadaic acid class and TPA-type tumor promoters, suggesting a difference in their natures (data not shown).

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Table 1 Western blot analysis of hyperphosphorylated cytokeratins with anti-cytokeratin antibodies

<table>
<thead>
<tr>
<th>Phosphoprotein</th>
<th>AE-3 (1–8)*</th>
<th>HK6 (6)</th>
<th>RCK 105 (7)</th>
<th>AE-1 (9–19)</th>
<th>MAK-6 (8,14–16, 18, 19)</th>
<th>PKK-2 (7, 16, 17, 19)</th>
<th>19 Ks (19)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>M, 60,000</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>CK 5</td>
</tr>
<tr>
<td>M, 58,000</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>CK 6</td>
</tr>
<tr>
<td>M, 56,000</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>CK 7</td>
</tr>
<tr>
<td>M, 52,000</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>CK 16</td>
</tr>
<tr>
<td>M, 42,000</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CK 19</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, specificity of cytokeratin peptide.

Fig. 2. Immunoprecipitation of hyperphosphorylated proteins in PHK 16-1 cells. The cells were treated with 100 nM dinophysistoxin-1 for 2 h. Immunoprecipitates were separated by 10% SDS-PAGE followed by autoradiography. Immunoprecipitates of cell lysates were treated with normal mouse serum (Lane 1), anti-cytokeratin antibody K8.13 (Lane 2), and anti-M, 27,000 heat shock protein antibody SPA-800 (Lane 3). kDa, kilodaltons.

Indirect Immunofluorescence Microscopy. PHK 16-1 cells (5 x $10^4$/chamber), plated on 4-chamber slides, were treated with 100 nM dinophysistoxin-1 for 15 min. After fixation of the cells with methanol, the slides were treated with the anti-cytokeratin antibody AE-3 for 2 h and further incubated with secondary antibody conjugated with fluorescein for 1 h. The slides were examined microscopically.

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Immunoprecipitation of Hyperphosphorylated Proteins. To test whether the hyperphosphorylated proteins were cytokeratins, the cell lysates were immunoprecipitated by the monoclonal anti-cytokeratin antibody K8.13, which mainly reacts with basic cytokeratins, and the immunoprecipitates were separated by SDS-PAGE (Fig. 2). The antibody K8.13 significantly precipitated phosphoproteins that had the same molecular weights as those in Fig. 1 (M, 60,000, M, 58,000, M, 52,000, and M, 42,000) but it did not precipitate the M, 27,000 protein. In addition, the antibody K8.13 immunoprecipitated a M, 56,000 phosphoprotein, which was later identified as cytokeratin 7 (Table 1), although the M, 56,000 phosphoprotein was difficult to distinguish in Fig. 1. Normal mouse serum did not significantly precipitate any phosphoproteins. The hyperphosphorylated M, 27,000 protein was immunoprecipitated by anti-M, 27,000, a heat shock protein, HSP27.
Hyperphosphorylated Cytokeratins by Okadaic Acid

Fig. 3. Two-dimensional gel electrophoresis of cytokeratins. The cells were treated with dimethyl sulfoxide as vehicle (A) or 100 nM dinophysistoxin-1 (B) for 2 h. The total cell lysates were subjected to two-dimensional gel electrophoresis followed by autoradiography. Numbers, cytokeratin peptides. IEF, isoelectric focusing.

Fig. 4. Hyperphosphorylation of cytokeratins in vitro. Cytokeratins obtained from PHK 16-1 cells were incubated with [32P]ATP, various concentration of dinophysistoxin-1 (1 to 1000 nM), and an enzyme fraction containing protein phosphatase 2A and protein kinases. Trichloroacetic acid-insoluble pellets were subjected to SDS-PAGE and autoradiography. Increased radioactivity of CK 5, CK 6, and CK 7 (○), CK 16 (■), and CK 19 (▲) were expressed as percentages. Points, mean of duplicate determinations.

(Fig. 2, Lane 3). These results showed that four of the phosphoproteins and the Mr 56,000 phosphoprotein are cytokeratins, but the Mr 27,000 protein is not.

Western Blot Analysis of Hyperphosphorylated Cytokeratins. To characterize the cytokeratin peptides, the soluble fraction of cell lysates was one-dimensionally separated and blotted onto nitrocellulose membranes, then subjected to Western blot analysis using seven anti-cytokeratin antibodies, as indicated in Table 1. According to specificities of the antibodies, phosphoproteins with molecular weights of 60,000, 58,000, 52,000, and 42,000 were identified to be CK 5, CK 6, CK 16, and CK 19, respectively (Table 1). In addition, the Mr 56,000 phosphoprotein reacted with the antibody RCK 105 against cytokeratin 7 (Table 1). CK 7 was not clearly visible in one-dimensional SDS-PAGE, due to the smaller amount of protein and a lesser amount of phosphorylation than those of CK 5 and CK 6.

Two-Dimensional Gel Electrophoresis of Cytokeratins. In this experiment, we used the total cell lysates because the hyperphosphorylated proteins had already been analyzed as described above. To confirm the cytokeratin peptide numbers, the gels were subjected to an autoradiography, not to Coomassie blue staining. The phosphoproteins of CK 5 and CK 6 were additionally separated into various spots, indicating different isoelectric points.

Fig. 3 also showed that phosphorylation of CK 5 and CK 6 was much stronger than that of CK 16 and CK 19 in the cells treated with dinophysistoxin-1. Hyperphosphorylated CK 7 was clearly separated in two-dimensional SDS-PAGE (Fig. 3).

Hyperphosphorylation of Cytokeratins in Vitro. Fig. 4 illustrated the increase of in vitro phosphorylation of cytokeratins from their basal levels, depending on the concentrations of dinophysistoxin-1, and expressed the increase of phosphorylation of the Mr 56,000-60,000 proteins, represented as cytokeratins CK 5, CK 6, and CK 7, the Mr 52,000 protein as CK 16, and the Mr 42,000 protein as CK 19. These results showed that in vitro hyperphosphorylated cytokeratins are potential substrates for protein phosphatase 2A.

Indirect Immunofluorescence Microscopy. The consequence of hyperphosphorylated cytokeratins was determined. An elaborated network of cytokeratin filaments was seen in the cytoplasm of the non-treated PHK 16-1 cells (Fig. 5A). Treatment with dinophysistoxin-1 for 15 min caused a significant retraction of cytokeratins from the cytoplasm into the perinuclear zone, a reaction probably associated with their hyperphosphorylation (Fig. 5B). However, the cells did not show any morphological changes at this stage. Further treatment with dinophysistoxin-1 induced morphological changes, such as bleb-like formations.
HYPERPHOSPHORYLATED CYTOKERATINS BY OKADAIC ACID

Tumor promoters of the okadaic acid class are potent inhibitors of protein phosphatases 1 and 2A, whereas the TPA-type tumor promoters are potent activators of protein kinase C (2, 15). Although their mechanisms of action are different, they induce tumor promotion on mouse skin initiated with DMBA with the same potencies (1). However, they commonly increase phosphoproteins in larger amounts than in the usual state. Our previous paper reported that vimentin, one of the intermediate filaments, is highly phosphorylated in primary human fibroblasts treated with okadaic acid (4). Similar results were recently reported with calyculin A (16). We first report that cytokeratins, one group of intermediate filaments, are also hyperphosphorylated in human keratinocytes by treatment with the okadaic acid class of tumor promoters. All of these results indicate that intermediate filaments are target proteins for hyperphosphorylation by the okadaic acid class of tumor promoters in various cells.

The cytokeratins include at least 19 individual cytokeratin peptides from CK 1 to CK 19 (7, 17). Expression of cytokeratins varies, depending on cell differentiation, states of transformation, and environmental factors (6, 7). PHK 16-1 cells used in the experiments contained mainly CK 5, CK 6, CK 7, CK 16, and CK 19. Basic cytokeratins CK 5 and CK 6 were phosphorylated much more than acidic cytokeratins CK 16 and CK 19 by dinophysistoxin-l, both in the cells and in cell-free systems.

Various serine/threonine protein kinases are thought to be involved in hyperphosphorylation of basic as well as acidic cytokeratins because vimentin and desmin are phosphorylated by cyclic AMP-dependent protein kinase, protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase, and p34^cdc2 kinase (18-20). However, it is not well known what kinds of protein phosphatases are involved in dephosphorylation of cytokeratins. Our results in Fig. 4 clearly showed that inhibition of protein phosphatase 2A by the okadaic acid class of tumor promoters induced \textit{in vitro} hyperphosphorylation of cytokeratins. The results were well correlated with the evidence that \textit{in vitro} hyperphosphorylation of vimentin was also obtained by inhibition of protein phosphatase 2A (4). Interestingly, three okadaic acid class tumor promoters (okadaic acid, dinophysistoxin-l, and calyculin A) have a similarly strong inhibitory activity toward protein phosphatase 2A, but not to protein phosphatase 1, and have similarly potent tumor promoting activity on mouse skin initiated with DMBA. Hyperphosphorylation of intermediate filaments induced by inhibition of protein phosphatase 2A seems to be well correlated with tumor promotion.

How phosphorylation of intermediate filaments is related to tumor promotion has not been well investigated. TPA is reported to induce reorganization of intermediate filaments in mouse epidermal cells and chicken embryo fibroblasts (21, 22). The transient phosphorylation of intermediate filaments by TPA might cause only temporary subcellular morphological changes. Intermediate filaments are also associated with microfilaments, microtubules and nuclear membranes, and phosphorylation of intermediate filaments causes effects which might be a reflection of signal transduction by a tumor promoter.

As for the nature of the $M_\text{s}$ 27,000 protein, it was thought to be a ribosomal S6 protein, based on studies of the phosphoprotein in isolated hepatocytes (23). However, using anti-$M_\text{s}$ 27,000 heat shock protein antibody, we identified HSP27 as a new member of the target proteins for the okadaic acid pathway. It is of interest to note that HSP27 is also phosphorylated \textit{in vitro} and in many cell lines treated with TPA (24). TPA and okadaic acid stimulate expression of various genes through the AP-1 complex binding site and various enhancer elements (25-27). We have recently demonstrated that okadaic acid induced hyperphosphorylation of tumor suppressor gene products such as retinoblastoma protein and p53 in human fibroblasts (28). The functional interaction between phosphorylated intermediate filaments and phosphorylation of transcription factors, along with phosphorylation of tumor suppressor gene products, should be further investigated.

REFERENCES


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