Apoptosis of squamous cells at different stages of carcinogenesis following 4-HPR treatment

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Abbreviations: 4-HPR, N-(4-hydroxyphenyl)retinamide; DiOC6, 3,3’-dihexyloxacarbocyanine iodide; HPV, human papilloma virus; mAb, monoclonal antibody; MPT, mitochondrial permeability transition; PI, propidium iodide; ROS, reactive oxygen species; SCC, squamous cell carcinoma; TPCK, N-tosyl-l-phenylalanil chloromethylketone.

Carcinogenesis vol.23 no.3 pp.447–456, 2002

Introduction

Squamous cell carcinoma (SCC) is the most frequent epithelial cancer and may occur at several body locations (1). It is the end product of a multistep carcinogenic process arising from preneoplastic lesions associated with chronic inflammation or, in certain sites (e.g. in the cervix), with infection by high-risk human papilloma virus (HPV) strains (2).

Low intake of vitamin A has been related to squamous cell cancer in epidemiological studies (3,4). Retinoids are vitamin A analogs and have been therefore studied as preventive and/or therapeutic drugs for squamous cell cancer. They were found to be potent inhibitors of carcinogenesis in the lung and the upper aerodigestive tract (5,6). However, retinoids generally produce a number of side effects that hamper their broad clinical application.

The synthetic retinoid N-(4-hydroxyphenyl)retinamide (4-HPR or fenretinide) has a low overall toxicity (7). It was shown to be an effective chemopreventive agent in animals (reviewed in ref. 8); moreover, its activity against human epithelial cancer was investigated in clinical trials, some of which are still in progress, with different results (9–16). Studies on in vitro cell models show that 4-HPR affects cell growth by inducing apoptosis, instead of exerting differentiative or cytostatic functions, as most retinoids do (17–19). This feature pertains to squamous carcinogenesis. In normal squamous epithelia an homeostatic equilibrium exists between proliferation and apoptosis of mature keratinocytes (20). Dysregulation of this program by aberrant differentiation and loss of ability to undergo physiological cell death may lead to the development of SCC. Thus, agents that restore the ability of pre-malignant and malignant squamous cells to undergo apoptosis may be useful for the treatment and prevention of SCC.

Few clues are presently available as for the mechanisms underlying the effects of 4-HPR. It may function by both retinoic acid receptor-dependent (21) and -independent (17) mechanisms. In leukemia and cervical cancer cells 4-HPR induces apoptosis by enhancing the production of reactive oxygen species (ROS) (18,22,23). It was also shown to induce apoptosis of leukaemia and neuroblastoma cells by a mechanism distinct from ROS production, such as an intracellular increase of the secondary messenger ceramide (24,25).

In the present work we studied the effects, and some effector mechanisms, of 4-HPR on squamous cells, with regard to a possible relationship with the stage of squamous carcinogenesis. This may be clinically relevant for better understanding and defining the timing of retinamide intervention during the carcinogenic process. We used an in vitro cell model of squamous carcinogenesis which represents sequential steps from normal squamous cells to advanced malignant stages. This model consists of normal human keratinocytes, keratinocytes showing a ‘dysplastic’ phenotype by transfection with the genome of the high-risk human papilloma virus type 16 (HPV-16), and fully transformed tumorigenic keratinocytes obtained by transfecting previously HPV-immortalized keratinocytes with the viral Ha-ras gene. This model provides an in vitro correlate of sequential progression stages from normal to dysplastic squamous epithelium and to SCC (26–28). In addition, we enriched the ‘neoplastic’ compartment of the model with three SCC cell lines. We found that 4-HPR affects...
cell growth in a dose-dependent fashion. At increasing 4-HPR concentrations, cells progressively underwent cell cycle perturbations (1.5–5 µM), followed by apoptosis (5–15 µM) and then necrosis (around 50 µM and above). The amount of apoptosis was very similar in normal keratinocytes as well as in the two transformed cell lines, suggesting that sensitivity to 4-HPR bears no relationship with the stage of squamous cell carcinogenesis. We further investigated the mechanisms involved in 4-HPR-mediated cell growth inhibition. We found that 4-HPR increases production of free radicals and induces mitochondrial permeability transition (MPT). MPT is an upstream event that mediates, in part, caspase 3 activity. In addition, 4-HPR activates caspases 8 and 9, and the former is not elicited through Fas–Fas ligand interactions.

Materials and methods

Cells, cell cultures and reagents

Human keratinocytes were obtained from skin biopsies and cultured on a lethally irradiated feeder-layer of 3T3 fibroblasts, as described (29). The non-tumorigenic UP cell line derived from keratinocytes immortalized by transfection with the E6 and E7 early genes of HPV-16, and the tumorigenic UPR cell line derived from UP by transfection with v-Ha-ras were kindly provided by Dr F.M.Watt (Imperial Cancer Research Fund, London, UK). Keratinocytes, UP and UPR cells were cultured in DMEM and Ham’s F12 media (3:1 mixture) containing FCS (10%), insulin (5 g/ml), transferrin (5 g/ml), adenine (0.18 mM), hydrocortisone (0.4 g/ml), cholera toxin (0.1 nM), L-ascorbic acid (C vitamin) and cyclosporin A from Sigma Chemical (St Louis, MO). The irreversible serine protease inhibitor TPCK was from Sigma Chemical. The irreversible caspase inhibitors Z-DEVD-FMK, Z-IETD-FMK and Z-LEHD-FMK (inhibitor of caspase 8 and 9) from Becton Dickinson (San Jose, CA) were added directly to the cell cultures at the concentrations indicated.

4-HPR (a gift of RW Johnson Pharmaceutical Research Institute, Springhouse, PA) was added directly to the cell cultures at the concentrations indicated.

†-ascorbic acid (C vitamin) and cyclosporin A from Sigma Chemical (St Louis, MO) were used as anti-oxidant and MPT inhibitor, respectively. As irreversible permeable inhibitors of caspases we used Z-DEVD-FMK (inhibitor of caspase 3 and, at much lower affinity, of caspases 6, 7, 8 and 10), Z-IETD-FMK (inhibitor of caspase 8 and granzyme B) and Z-LEHD-FMK (inhibitor of caspase 9 and, at lower affinity, of caspases 4 and 5) (Calbiochem, San Diego, CA). The irreversible serine protease inhibitor N-tosyl-L-phenylalanyl chloromethylketone (TPCK) was from Sigma Chemical. The agonist apoptosis-blocking anti-Fas mAb (ZB4, IgG1) was from Upstate Biotechnology (Waltham, MA). Inhibitors were added to the cell cultures 3 h prior to 4-HPR treatment. Inhibitor doses were chosen according to the concentrations that significantly inhibited apoptosis induced by positive control drugs, namely agonist apoptosis-inducing anti-Fas antibody CH11 (IgM, from Upstate Biotechnology, Waltham, MA) for caspase 8-inhibitor, caspase 3-inhibitor and ZB4; cisplatin (Sigma Chemical) for caspase 9-inhibitor; and camptothecin (Sigma Chemical) for TPCK. We found that effective inhibitory concentrations were 15 µM, 15 µM and 5 µM for the caspase inhibitors Z-DEVD-FMK, Z-IETD-FMK and Z-LEHD-FMK, respectively, 50 µM for TPCK and 2 µg/ml for ZB4.

The anti-Fas ligand mAb (NOK-1, IgG1), used to evaluate by immunofluorescence the expression of Fas-ligand on 4-HPR-treated squamous cells, was from Becton Dickinson (San Jose, CA). As a positive control we used the HL-60 cell line that bears surface Fas ligand molecules.

DNA flow cytometry for apoptosis and cell proliferation evaluation

Cells were harvested and fixed with 70% ethanol for 48 h at −20°C. After washing with PBS, samples were incubated with 30 µg/ml propidium iodide (PI) (Sigma Chemical) and 0.5 mg/ml RNase for 30 min at room temperature and in the dark. Flow cytometric measurements were performed using a FACSCalibur (Becton Dickinson) equipped with a 488 nm laser for PI excitation and optical filters for PI emission fluorescence. At least 10 000 events were acquired on a linear scale from each sample. Frequency distributions of DNA content (DNA histograms) were analyzed for the evaluation of apoptosis and percentage of cells in the various phases of the cell cycle, as described (30).

Analysis of annexin V binding by flow cytometry

Cells were treated with annexin V-FITC (Becton Dickinson) and PI for 15 min at room temperature, according to the manufacturer instructions. FITC and red PI fluorescences were measured using a FACSCalibur flow cytometer on logarithmic scales and analyzed as bivariate dot plots. FITC- and PI-double negative are viable cells, FITC-positive and PI-dimly positive are apoptotic cells (externalized phosphatidylserine residues and slightly increased cell membrane permeability due to apoptosis), whereas FITC-positive and PI-brightly positive are late apoptotic/necrotic cells. For each sample, 10 000 events were acquired on a logarithmic scale for both FITC and PI fluorescence.

Propidium iodide uptake test

PI uptake tests were performed to assess cell viability vs. cell death. Briefly, cells were treated for 5 min with 3 µg/ml PI and analyzed by flow cytometry. Cells that did not take up the fluorochrome (i.e. negative fluorescence) were considered as viable, whereas fluorescent cells, i.e. cells that took up the dye due to damaged plasma membrane, were considered as dead (late apoptotic + necrotic cells). At least 10 000 events were acquired on a logarithmic scale from each sample.

Detection of MPT

Induction of MPT was evaluated by the decrease in mitochondrial inner membrane potential as described (31). Briefly, cells were incubated with...
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Fig. 2. 4-HPR induces cell cycle perturbations at lower doses and apoptosis at higher doses in A431 and LX-1 SCC cell lines. Flow cytometric DNA content distributions of A431 and LX-1 cell lines treated for 48 h with 4-HPR at the reported concentrations. Arrows in the first histogram show the regions corresponding to apoptotic cells (‘Apo’, in the sub-G1 region), G0/G1 cells (‘G1’), cells in S phase (‘S’) and G2/M cells (‘G2/M’). At 1.5 µM and 5 µM a reduced G1/S transit rate occurs in A431 cells, as revealed by the decrease of the cell percentage in S phase. Conversely, at the same doses, LX-1 cells accumulate in the G2/M phase. At 4-HPR concentrations of 15 µM and above a significant rate of apoptosis occurs.

40 nM 3,3'-dihexyloxolacarbocyanine iodide (DiOC6) for 15 min at 37°C and analyzed by a FACSCalibur equipped with a 488 nm laser for DiOC6 excitation and optical filters for DiOC6 emission fluorescence (552 nm). At least 10 000 events were acquired per sample. The suppression of transmembrane potential causes a shift of DiOC6 fluorescence to lower levels.

Caspase 3 activity
Caspase 3 activity was assayed as described (25). Briefly, cell lysates were prepared by resuspending the cell pellet in hypotonic lysis buffer (50 µl/10⁶ cells) containing 10 mM HEPES (pH 8.0), 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 10 µg/ml each of aprotinin, leupeptin, and pepstatin. Cell suspensions were incubated on ice for 15 min, passed five times through a 27-gauge needle, and centrifuged at 14 000 r.p.m. for 15 min at 4°C. Cell lysates (50 µg) were added to 148 µl of reaction buffer (100 mM HEPES (pH 7.5), 20% glycerol, 0.5 mM EDTA, and 5 mM DTT) and 2 µl of substrate Ac-DEVD-pNA (Calbiochem) (final concentration, 100 µM), followed by incubation at 30°C for 6 h. The enzyme-catalyzed release of pNA was monitored at 405 nm in a microtiter plate reader (Titertek Multiskan PLUS, Flow Laboratories, Ayrshire, UK).

Results
4-HPR affects squamous cell growth by inducing apoptosis
The effects of 4-HPR on squamous cells were studied by measuring cell growth and cell death 48 h after drug treatment at different concentrations. An in vitro model of squamous cell carcinogenesis was used and consisted of primary cultures of normal keratinocytes, of a non-tumorigenic HPV-immortalized keratinocyte line (UP), and a tumorigenic HPV-immortalized/v-Ha-ras-transfected keratinocyte line (UPR). This model has been shown to represent an in vitro equivalent of progressing stages from normal squamous epithelium (normal keratinocytes) to dysplastic (UP cells) and to SCC (UPR cells) (26,27). In addition to the normal and transformed keratinocytes, we studied three SCC cell lines (LX-1, HeLa and A431).

Cell numbers in 48 h-treated cultures indicated a dose-dependent inhibition of cell growth by 4-HPR (Figure 1A). Analyses of PI uptake showed increasing cell death at increasing 4-HPR concentrations (Figure 1B). The response to the drug was very similar in all cell types tested (Figure 1). In particular, no difference was found between normal, immortalized and tumorigenic keratinocytes, suggesting that no relationship exists between sensitivity to 4-HPR and stage of squamous cell carcinogenesis of our in vitro model.

Flow cytometric DNA measurements for the evaluation of possible effects of 4-HPR on the cell cycle were performed. DNA histograms of A431 and LX-1 cells are shown in Figure 2. At 1.5 µM and, more markedly, at 5 µM, a reduced G1/S transit rate occurs in A431 cells, as indicated by the decrease of the cell percentage in S phase. Conversely, LX-1 cells were induced to arrest in the G2/M phase of the cell cycle, as indicated by the increase of the G2/M cell fraction (Figure 2 and Table I). Normal keratinocytes, UP and UPR cells, as well as the SCC cell line HeLa, were not shown in the figure because they did not exhibit cell cycle perturbations at such 4-HPR concentration (Table I). At higher concentrations, i.e. 15 µM and above, 4-HPR induced cell death by apoptosis in all cell types, as suggested by the presence of cells in the sub-G1 region of the DNA histograms (Figure 2 and not shown). Following these data, concentrations of the drug within the 5–15 µM range were studied, and flow cytometric DNA measurements were performed also at 10 µM 4-HPR. As shown
Table I. Effects of 48 h 4-HPR treatment on cell cycle distributions, apoptosis and mitochondrial membrane permeability transition (MPT)

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<th>MPT</th>
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<td>S (%)</td>
<td>G2/M (%)</td>
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*aPercentage of annexin V positive cells, both PI negative and positive.

Fig. 3. 4-HPR induces cell death in squamous cells. Squamous cells were treated for 48 h with 10 µM 4-HPR and flow cytometric annexin V-FITC/PI measurements were performed. FITC- and PI-double negative are viable cells, FITC-positive and PI-dimly positive are apoptotic cells (increased cell membrane permeability due to apoptosis), whereas FITC-positive and PI-brightly positive are late apoptotic/necrotic cells.

in Table I, apoptosis was observed at the latter concentration for all cell types (‘sub-G1 region’ in Table I).

To further confirm apoptotic cell death, 4-HPR-treated cells were analyzed for their reactivity with annexin V. Annexin V-FITC/PI dot plots are shown in Figure 3 (for all cell types tested) and Figure 4A (for UP and UPR cells). The results, reported also in Table I (sixth data column), display a trend very similar to that shown by the DNA content histograms, although the percentages of annexin V-positive cells are generally higher than those of the sub-G1 cell fraction. Accord-
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Fig. 4. 4-HPR induces apoptosis and MPT in squamous cells. UP and UPR cells were treated for 48 h with 4-HPR (5 µM or 10 µM) and/or cyclosporin A (5 µM, added 3 h prior to 4-HPR). (A) Flow cytometric annexin V-FITC/PI dot plots. FITC- and PI-double negative are viable cells, FITC-positive and PI-dimly positive are apoptotic cells (increased cell membrane permeability due to apoptosis), whereas FITC-positive and PI-brightly positive are late apoptotic/necrotic cells. (B) Flow cytometric DiOC6 fluorescence histograms. Fluorescence of this dye is a function of the mitochondrial inner membrane potential. Decreased fluorescence (highlighted by the marker) indicates reduction of mitochondrial inner membrane potential, i.e. mitochondrial permeability transition (MPT). The percentage of cells undergoing MPT is reported. Cyclosporin A inhibits 4-HPR induced MPT.

Mitochondrial permeability transition (MPT) occurs in 4-HPR-treated squamous cells

Increased production of reactive oxygen species (ROS) has been reported as a central mechanism of 4-HPR-induced apoptosis (18,22). In our observations, a 3 h pretreatment with the anti-oxidant C vitamin partially rescued cells from apoptosis, as shown in Figure 5 (third row) for UP and UPR cells analyzed for viability (PI uptake histograms) and apoptosis (DNA content distributions). Since increased production of ROS is associated with mitochondrial depolarization (32), we asked whether MPT was involved in 4-HPR-induced apoptosis. MPT is due to opening of mitochondrial channels that can be triggered by ROS, and results in a dissipation of the mitochondrial inner membrane potential. MPT was measured as decreased fluorescence of DiOC6, a lipophylic cationic probe that localizes in the mitochondria of viable cells because of the relatively high electric potential across the mitochondrial inner membrane. The DiOC6 fluorescence histograms shown in Figure 4B indicate that a loss of mitochondrial membrane potential occurs in a proportion of cells at 5 µM 4-HPR and involves the large majority of them at 10 µM drug concentration. The data for the other squamous cells are summarized in Table I (seventh column), and are expressed as percentages of cells with decreased DiOC6 fluorescence. The results demonstrate that 10 µM 4-HPR induces MPT in a very high proportion of cells. Moreover, comparison of data among the three keratinocyte cell types indicates that no relationship exists between MPT and stage of squamous carcinogenesis.

Cyclosporin A inhibits MPT (31,32). Accordingly, pretreatment of squamous cells with cyclosporin A rescued cells from 4-HPR-induced MPT (Figure 4B). To further assess the role of MPT in 4-HPR-induced apoptosis, we measured DNA content for the evaluation of apoptotic cells and PI uptake to detect late apoptotic/necrotic cells. We found that the percentage of apoptotic and necrotic cells in 4-HPR + cyclosporin A-treated cultures was lower than in cultures treated with 4-HPR alone (Figure 5). This suggests that MPT is involved in 4-HPR-induced cell death. However, inhibition was only partial, as shown by values reported in Figure 5 and by data obtained with other squamous cell lines (not shown). This is possibly due to the presence of additional death effector pathways not involving MPT.

Caspases 8, 9 and 3 are involved in 4-HPR-mediated apoptosis of SCC cells, and MPT is upstream caspase 3 activation

The effect of caspase inhibitors on 4-HPR-treated UP, UPR and A431 cells was studied by adding, 3 h before 4-HPR, specific inhibitors of caspases 3, 8 and 9. Inhibitors significantly decreased 4-HPR-induced apoptosis and necrosis, but not MPT, as shown in Figure 6 for UPR cells. These data indicate that
caspases are involved in the 4-HPR death effector pathway, and suggest that MPT precedes caspase activation.

We measured caspase 3 activity in 4-HPR + cyclosporin A-treated cultures of UP and UPR cells, and found that it was lower than in cultures treated with 4-HPR alone (Figure 7). This reinforces the role of MPT in regulating caspase activation. However, inhibition was not complete, again suggesting that other mechanisms of caspase 3 activation may be elicited by 4-HPR.

We also investigated whether 4-HPR required activation of serine proteases to induce MPT and apoptosis. To this end we pretreated UP, UPR and LX-1 cells with the serine protease inhibitor TPCK for 3 h before 4-HPR addition, at the concentration of 5 µM, that we found to optimally inhibit camptothecin-induced apoptosis. TPCK did not reduce MPT and apoptosis triggered by 4-HPR, as revealed by flow cytometric DiOC₆ fluorescence (Figure 8 for LX-1 cells) and DNA content histograms (not shown), respectively.

4-HPR-mediated MPT and apoptosis in SCC cells are not elicited by Fas–Fas ligand interactions

Because caspase 8 has been implicated as a regulator of apoptosis mediated by Fas receptors, we further investigated a putative Fas involvement in 4-HPR-induced apoptosis. To this end, we measured the surface expression of Fas ligand by anti-Fas ligand antibodies and flow cytometry in UP, UPR and LX-1 cells, and found no detectable surface protein level, neither before nor after 4-HPR treatment, at variance from HL-60 cells which represented the positive control (not shown). In addition, we inhibited possible interactions between Fas and Fas ligand molecules by the antagonist anti-Fas antibody ZB4, which was added 3 h before 4-HPR. We observed the same rate of apoptosis in 4-HPR- and in 4-HPR + ZB4-treated cell cultures (not shown). In addition, 4-HPR-induced MPT was not affected by ZB4 (Figure 8). These data indicate that neither 4-HPR-mediated apoptosis, nor MPT, are elicited by the Fas pathway.
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Discussion

SCC is the end product of a multistep and field carcinogenic process that involves the progression from normal epithelial cells, through metaplastic or dysplastic squamous epithelia, resulting in fully transformed, invasive cancer (1). The preneoplastic lesions are associated with a history of chronic inflammation or, in certain sites (e.g. in the cervix), with infection with high-risk HPV types (2). The synthetic retinoid 4-HPR has been proposed for the treatment of SCC, based on its chemopreventive and/or therapeutic activity observed in experimental animals (reviewed in ref. 8) and in human clinical trials (13,16,52). However, it is not clear whether 4-HPR is more effective as chemopreventive drug in reversing squamous metaplasia or dysplasia (15,16,33,52), or as a therapeutic drug in destroying fully transformed squamous cancer cells (34–37). In vitro systems of squamous cell carcinogenesis may be useful to shed light on this issue. As mentioned, HPVs, particularly HPV-16 and HPV-18, play an important role in the squamous carcinogenic process. In addition, activation of the Ha-ras gene has been observed in several invasive cervical carcinomas (38). Based on these observations, in vitro systems have been developed that employ human epithelial cells converted to a dysplastic and then to a neoplastic phenotype by sequential transfections with specific HPV and ras DNAs (26,27,39). In the present study two transformed keratinocyte cell lines were used, i.e. UP cells, that are HPV-16 transfected keratinocytes (‘dysplasia’) and UPR cells, that are UP cells further transfected with v-Ha-ras (‘neoplasia’). Together with primary cultures of normal keratinocytes (‘normal tissue’), these cell lines provide a model of squamous carcinogenesis in vitro (26,27).

The effects of 4-HPR on each of the three cell types were studied. 4-HPR induced a dose-dependent cell death by apoptosis, which was associated with depolarization of mitochondrial membranes. However, these effects were very similar in the three keratinocyte types, suggesting that no relationship exists between sensitivity to the drug and stage of squamous carcinogenesis in our in vitro model. Accordingly, in a previous study it was shown that the sensitivity of in vitro cultured normal human bronchial epithelial cells and non-small cell lung carcinoma cell lines to 4-HPR was similar (40). Conversely, other studies demonstrated that 4-HPR was
less effective in inducing apoptosis of normal cervical and oral epithelial cells than of cervical carcinoma and SCC cells of the oral cavity, respectively (18,41). The contradiction of these data could be due to different levels of growth factors and mitogenic substances in the culture media of the normal cells utilized in the different studies.

That normal cells of our in vitro model are as sensitive to 4-HPR as transformed cells, may not be desirable for in vivo applications. However, we cannot extrapolate our in vitro data to in vivo situations. Importantly, no acute or severe toxicity was observed in 4-HPR-treated patients which could be associated to a significant damage of normal tissues (7,53). Long-term (5 years) results from a clinical trial including 2867 patients show that the main side effects of 4-HPR were impaired dark adaptation (cumulative incidence, 19%), due to the drug-induced decline of plasma retinol levels, and mild-grade dermatologic disorders (18.6%) (7). These symptoms tended to recover after the first year of treatment (7). In addition, measurements of 4-HPR in the plasma of patients receiving the usual dose of 200 mg p.o. per day, showed that serum levels are ~0.3–1 µM (15,42) and remain constant through a period of 5 years (53). At these low concentrations 4-HPR did not affect the in vitro growth of our squamous cell lines. On the other side, it is desirable that 4-HPR reaches toxic levels in tumor tissues. Evaluation of 4-HPR concentrations in breast biopsies of patients treated with 4-HPR before surgery, confirmed the ability of this retinoid to accumulate in the breast, with peak concentrations of ~10 µM (43). No other data on the drug distribution in tissues are so far available.

To analyze the mechanisms of 4-HPR-mediated apoptosis, we used transformed keratinocytes, i.e. UP and UPR cells. In addition, three tumor cell lines derived from SCCs of different regions were used (LX-1 from the lung, HeLa from the cervix and A431 from the vulva). We found that after 4-HPR treatment, the same mechanisms were operational in UP and UPR cells, as well as in the three SCC cell lines. This suggests that the neoplastic transformation of dysplastic squamous cells does not interfere with the pathways used by 4-HPR for apoptosis induction.

Caspases are involved in apoptosis by 4-HPR. These cysteine proteases play a critical role in the execution phase of apoptosis and are responsible for many of the biochemical and morphological changes associated with apoptosis (44). Caspases 8 and 9 are considered as ‘initiator’ caspases for two independent pathways, the death-receptor- and the mitochondrial-pathways, respectively, and have been shown to activate common downstream effector caspases, such as caspase 3 (44). The triggering of Fas by Fas ligand in the death-receptor pathway induces the formation of DISC, consisting of Fas, a Fas-associated protein with a death domain (FADD/MORT1) and caspase 8 (45). Upon recruitment into DISC, caspase 8 is activated by proteolytic cleavage, and propagates the death signal by triggering other caspase activation events. In the mitochondrial pathway, disruption of the inner transmembrane potential leads to release of intermembrane proteins through the outer membrane, such as cytochrome c and caspase 9 (46). Binding of cytochrome c, released in the cytosol, to the adaptor protein

![Fig. 7. Caspase 3 activity is partially prevented by MPT inhibitor. UP and UPR cells were treated for 48 h with 4-HPR (10 µM) in the absence or in the presence of cyclosporin A (5 µM). The inhibitor was added 3 h prior to 4-HPR. Caspase 3 activity was measured by a colorimetric assay (see Materials and methods). Values of the treated samples are expressed as fold increase in comparison with untreated control cultures. Results are expressed as the mean ± range/2 from two independent experiments.](image)

![Fig. 8. 4-HPR-dependent MPT is not reduced by inhibitors of serine proteases nor of Fas signalling. LX-1 cells were treated for 48 h with 4-HPR (10 µM), in the absence or in the presence of (A) the serine protease inhibitor TPCK (5 µM) and (B) antagonist apoptosis-blocking anti-Fas antibody ZB4 (2 µg/ml). The concentration of the two inhibitors was selected according to their effectiveness in positive controls, i.e. against apoptosis induced by (A) topoisomerase I inhibitor Camptothecin (CAM, that activates serine proteases) and (B) anti-Fas antibody CH11 (that simulates Fas–Fas ligand interactions). Data are shown as flow cytometric dot plots of DiOC6 fluorescence vs. forward light scatter (FSC). Cells displaying reduced FSC and DiOC6 fluorescence are undergoing MPT.](image)
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In conclusion, the aim of the present work was to highlight a clinically relevant issue, namely the timing of retinamide intervention during the carcinogenic process. To this end, we studied the activity of 4-HPR on squamous cells with respect to the stage of squamous carcinogenesis. We demonstrate that 4-HPR exerts the same cytotoxic activity in vitro on dysplastic and neoplastic squamous cells, at doses which could be found in vivo in dysplastic or neoplastic tissues of patients undergoing 4-HPR treatment.

Acknowledgements
This study was supported by grants from Associazione Italiana Ricerca sul Cancro (AIRC) and from Ministero Università e Ricerca Scientifica e Tecnologica (MURST) to C.E.G. and E.C. We are grateful to Dr Andrea Decensi (Division of Chemoprevention, European Institute of Oncology, Milan, Italy) for helpful discussions throughout the study.

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