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Evidence for Abrogation of Oncogene-induced Radioresistance of Mammary Cancer Cells by Hexadecylphosphocholine *in vitro*

E.A. Bruyneel, G.A. Storme, D.C.C. Schallier, D.L. Van den Berge, P. Hilgard and M.M. Mareel

Hexadecylphosphocholine (HePC), an experimental and clinical antitumour agent of the alkyllysophospholipid group, was tested for its radiosensitising effect on a panel of nine human mammary cancer cell lines *in vitro*. Growth inhibition by ionising radiation and recovery from it were not influenced by pretreatment with HePC in most cases, except for two cell lines expressing an activated *ras* oncogene. In the latter we found an enhanced radioresistance that was abolished by pretreatment with HePC. Our results suggest that HePC may act as a radiosensitiser for cells carrying an activated *ras* oncogene.
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INTRODUCTION

HEXADECYLPHOSPHOCHOLINE (HePC), an alkylphosphocholine lacking the glycerol backbone of alkyllysophospholipids, has antitumour activity as tested on a number of rodent and human tumour cell lines in culture [1]. Furthermore, it inhibits the growth of experimental tumours in the rat [2] and clinical studies have demonstrated regression of small skin nodules in breast cancer patients after topical application of HePC [3].

HePC is known to inhibit protein kinase C (PKC) and PKC modulators may change the effect of cytotoxic agents on cancer cells [4]. Whether or not HePC also changes the effect of ionising radiation (I.R.) has not been studied. Potential radiosensitising agents are actively searched for, especially if they are expected to have some specificity for cancer cells as compared with normal cells.

To study combined effects of I.R. and HePC, we have chosen from well-defined cell families, variants that showed discrete phenotypic differences in hormone receptors, invasiveness, oncogene expression and differentiation markers. Mammary cells, mostly of human origin, were chosen because of the documented induction of differentiation in experimental mammary tumours by HePC [5]. The choice did not take into account *ras* oncogene expression, because this was, at that time, not supposed to be a crucial event. Later, it became obvious that activation of *ras* genes led to enhanced radiation resistance [6–9].

Effects of treatment on growth of cell populations were analysed with assays permitting cell–cell interactions in spheroids as described previously [10, 11]. For cells that did not form spheroids a modified assay procedure was developed. Emphasis was on recovery from growth inhibition rather than on cell killing or growth arrest.

Our results demonstrate a radiosensitising effect of HePC for some mammary cancer cell types and suggest that this effect might be related to the expression of an activated *ras* oncogene.

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MATERIALS AND METHODS

Cells

We have selected variants of different mammary cancer cell lines because of documented differences in a variety of characteristics. MCF-7/AZ and MCF-7/6 cells are, respectively, non-invasive and invasive *in vitro* [12]. MCF-7pneo*ras*TD5 cells express the v-Ha-*ras* oncogene, in contrast with their parental MCF-7/AZ cells [13]. Evsa-T is an MCF-7 variant that expresses at high levels the progesterone receptor [14]. HBL-100HP cells were chosen as a prototype of myoepithelial cells that underwent apparently spontaneous conversion towards malignancy [15]. SK-BR-3 cells express at high levels the *neu* oncogene product p185$^{c\text{-}erbB2}$ [16]. MDA-MB-435S/1 cells lack the cell–cell adhesion molecule E-cadherin; they were transfected to express the exogenous mouse E-cadherin gene and coined MDA-MB-435S/1CAD/B1B4. The MDA-MB-435S/1neo-B2 cell line serves as a neomycin-resistant control transfectant [17]. To examine further a possible role for the *ras* oncogene product in resistance to HePC and I.R., we used the *ras*-transfected mouse mammary cell line NMpneoT24T10 (kindly provided by K. Vleminckx and F. Van Roy, Laboratory of Molecular Cell Biology, University of Ghent, Belgium). This and other members of the NM family have been described previously [11, 18]. Cell lines were maintained on tissue culture plastic substrate with their appropriate media.

Treatments

Cells that produced spheroids in suspension culture were irradiated as such after selection for a diameter of 0.2 mm. Cells that did not produce spheroids were irradiated as confluent cultures on the solid tissue culture substrate at room temperature and under ambient air pressure before harvesting for cell survival

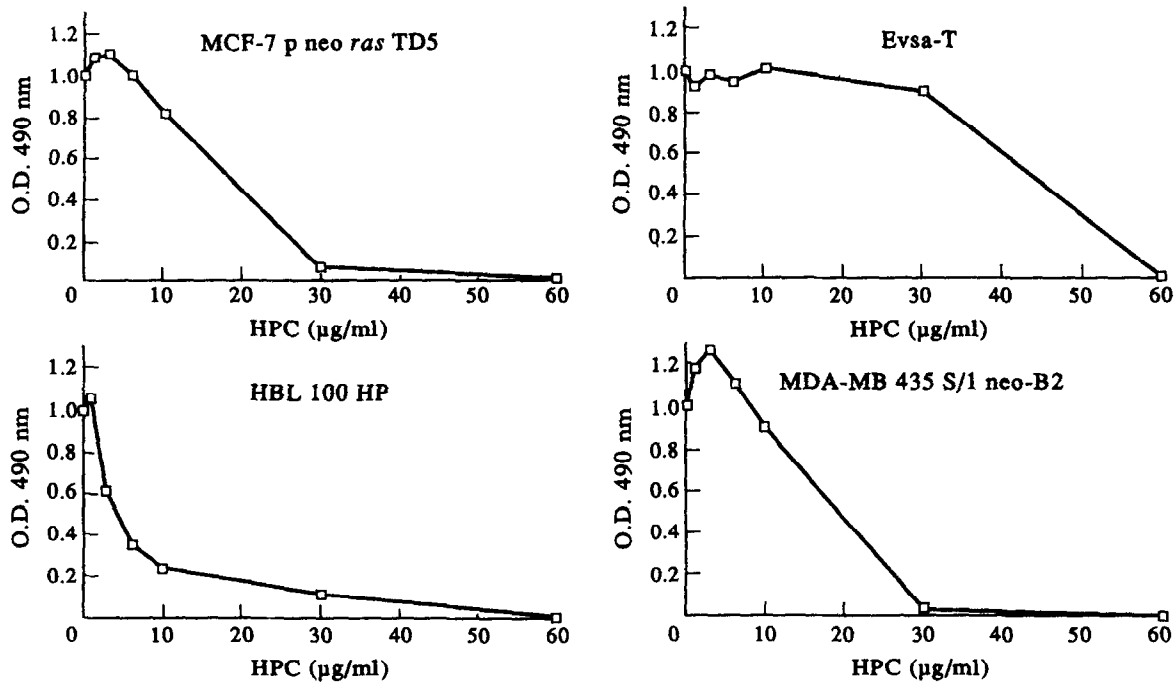

Fig. 1. Dose-dependent decrease in cell survival after treatment with HePC. Ordinate, optical density (O.D.) as measured in the MTT test; mean values from eight cultures, normalised to 1.0 for untreated cultures. Abscissa, concentration of HePC (μg/ml).

and growth assays. For I.R., we used photons produced by a ^{60}Co Gammatron III (Siemens, Erlangen, F.R.G.) at 70 cGy/min. Absorbed doses, as measured with lithium fluoride thermoluminescent powder (TLD7300; Teledyne Isotopes, Westwood, New Jersey, U.S.A.) were within the expected ranges ± 2 %.

HePC was obtained from ASTA Medica (Frankfurt, F.R.G.); it was diluted from a stock solution (3 mg/ml culture medium) at concentrations between 1 and 60 μg/ml. HePC was added to the spheroids or to cells in culture on solid substrate 48 h before the onset of the growth assays. In case of combined treatment, the sequence was as follows: (i) subconfluent cultures on solid substrate were treated with HePC or untreated for 48 h, then the confluent cultures were either irradiated or left untreated, and finally single cell suspensions were prepared and used to seed 24-well plates. (ii) Spheroids in shaker flasks were treated with HePC or untreated for 48 h, then selected spheroids (diameter = 0.2 mm) were irradiated or left untreated before explantation in a 24-well plate.

Table 1. Selection of treatment doses for combinations of ionising radiation and HePC

Cell type	HePC LD_{50} (μg/ml)	HePC LD_0 (μg/ml)	I.R. ID_{0-20} (Gy)
MCF-7/AZ	17.3	6.0	10
MCF-7/6	36.3	10.0	6
MCF-7pneo*ras*TD5	17.8	6.0	14
Evsa-T	43.2	30.0	6
HBL-100HP	4.2	3.0	10
MDA-MB-435S/1	8.9	3.0	10
MDA-MB-435S/1CAD/B1B4	21.5	10.0	10
MDA-MB-435S/1neo-B2	19.5	10.0	12
SK-BR-3	20.8	10.0	10
Average	21.1	9.8	9.8

LD_{50}, concentration of HePC allowing survival to 50% of untreated cultures in MTT test. LD_0, maximum concentration of HePC allowing survival equal to untreated cultures. ID_{0-20}, maximum dose of ionising radiation allowing growth after 2 days in at least 80% of cultures.

Assay for cell survival

The 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium test (MTT test) was conducted in accordance with a protocol published by Romijn [19] and as used by us in previous studies with HePC [11]. Cells were cultured in 96-well microtitre plates (Nunc, Roskilde, Denmark) and HePC was added to the culture during the last 7 days. The mean optical density (O.D.) of solubilised formazan was measured at day 8 in eight cultures per concentration of HePC and reported as a function of increasing HePC concentration.

Assay for growth

The choice of the assay for growth depended on whether or not cells formed multicellular spheroids in suspension culture. Individual spheroids with a diameter of 0.2 mm were explanted in 24-well plates as described above. The largest and its perpendicular diameter were measured and used to calculate the surface of the area covered by the cells. Increase in surface area beyond 5 mm^2 was considered as an index of growth, because such an area could not be covered by migration of cells from the explant without growth [10].

For cell types that did not form spheroids, droplets of 15 μl of single cell suspensions at 1.6 × 10^6 cells/ml were seeded on to tissue culture substrate and 1 ml of medium was added 24 h later. Suspensions were prepared by trypsinisation within 1 h after I.R. With such cultures, one or more of the following indices were scored as positive for growth: saturation density within the area of seeding; clonal outgrowth within the area of

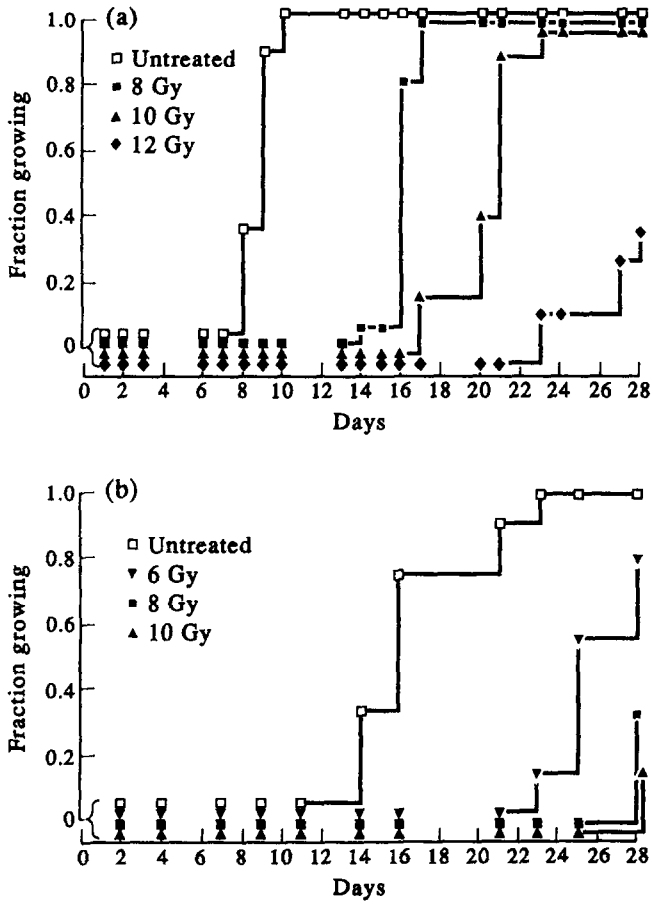

Fig. 2. Effect of ionising radiation on the growth of spheroids of MCF-AZ (panel a) and MCF-7/6 (panel b) cells explanted on tissue culture substrate. Ordinate: fraction of cultures positive for growth (area covered over 5 mm²). Abscissa: time after explantation of spheroids.

seeding; radial outgrowth leading to an increase of the area covered by cells. In all experiments, eight to 12 cultures were used for each treatment. Incubations were at 37°C in an atmosphere of air plus 10% or 5% CO_2 and with 100% humidity for at least 28 days. Media were replenished weekly.

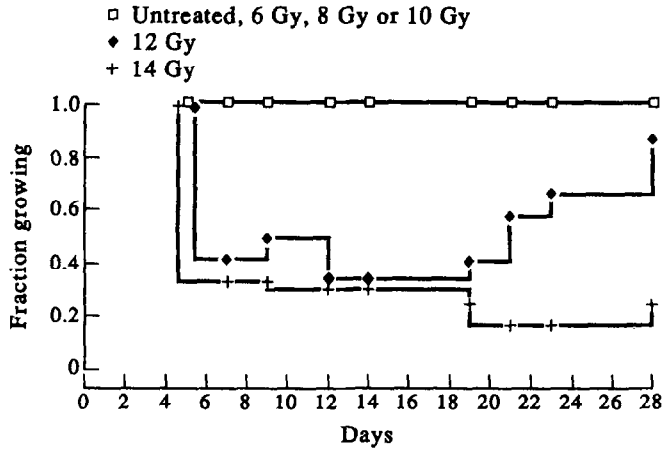

Fig. 3. Illustration of the type of growth curves obtained with MDA-MB-435S/1neo-B2 cells seeded from suspension on tissue culture substrate, untreated or after irradiation. Ordinate: fraction of cultures showing growth; first score after 5 days. Abscissa: days after seeding.

RESULTS

Effect of HePC on cell survival

A dose-dependent decrease in O.D. was observed for all cell types at concentrations between 6 and 60 μg/ml. Typical curves are shown in Fig. 1. For most cell types, with the exception of SK-BR-3 cells, MDA-MB-435S/1CAD/B1B4 cells and Evsa-T cells, a slight increase in O.D. was observed at concentrations of 1 and 5 μg/ml. The concentration of HePC that produced a 50% decrease in O.D. (LD_{50}) varied for the different cell types between 4 and 43 μg/ml (Table 1).

Effect of I.R. on growth

In experiments with spheroids the fraction of cultures meeting the criterium for growth (area covered of at least 5 mm²) after 28 days was lower with higher doses of I.R. (Fig. 2). This figure also shows that the delay in reaching the 5-mm² limit increases with increasing doses of I.R. In experiments with cell suspensions, the fraction of cultures which recovered from growth inhibition was also lower with higher doses of I.R. (Table 2). Furthermore, higher doses corresponded with an earlier growth arrest and a greater maximum inhibition of growth, as seen by the lower fraction of cultures showing growth arrest (Table 2, Fig. 3). For combined treatments we chose doses of I.R. that

Table 2. Growth characteristics of irradiated cell populations seeded as droplets of single cells on tissue culture substrate

Cell type	Day of growth arrest*								Day of recovery†			
	Onset				Maximum growth inhibition							
	8 Gy	10 Gy	12 Gy	14 Gy	8 Gy	10 Gy	12 Gy	14 Gy	8 Gy	10 Gy	12 Gy	14 Gy
MCF-7/AZ		5 (0.8)				19 (0.2)				23 (0.5)		
HBL-100HP	no	no	9 (0.7)	7 (0.0)	no	no	16 (0.3)	7 (0.0)	no	no	21 (0.5)	nr (0.0)
MDA-MB-435S/1	no	no	16 (0.5)	7 (0.2)	no	no	21 (0.1)	19 (0.0)	no	no	nr (0.1)	nr (0.0)
MDA-MB-435S/1CAD/B1B4	no	12 (0.9)	5 (0.7)	5 (0.7)	no	12 (0.9)	7 (0.0)	7 (0.0)	no	14 (1.0)	nr (0.0)	nr (0.0)
MDA-MB-435S/1neo-B2	no	no	7 (0.4)	7 (0.3)	no	no	12 (0.3)	21 (0.3)	no	no	19 (0.4)	28 (0.3)
SK-BR-3	no	no	19 (0.6)	7 (0.5)	no	no	21 (0.4)	19 (0.1)	no	no	nr (0.4)	nr (0.1)

* Figures in parentheses are the fraction of cultures showing arrest of growth. † Figures in parentheses are the fraction of cultures showing growth, with or without previous arrest. no, no growth arrest observed. nr, no recovery of growth inhibition observed.

resulted in growth of at least 80% of the cultures after 28 days (ID_{0-20} in Table 1).

Combinations of HePC and I.R.

Doses of HePC used in combined treatments were chosen from MTT tests, except for NMpneoT24T10 cells, for which we used preliminary data from spheroid cultures. In both cases the selection aimed at minimal effect of either treatment alone. In experiments with spheroids, treatment with I.R. following HePC, at LD_0 (see Table 1) had little or no effect on growth, except for *ras*-transfectant MCF-7pneo*ras*TD5 cells (Table 3). The results with MCF-7/6 cells even suggested some protective effect of HePC. For the *ras*-transfectant, the combined treatment resulted in complete inhibition of growth without any recovery at day 28. Absence of recovery from growth inhibition was also found in experiments with suspensions of MCF-7pneo*ras*TD5 cells (Fig. 4). For all other cell types tested by seeding as a suspension, the combination of HePC plus I.R. produced no growth delay, as with treatment with either agent alone (data not shown).

Combined treatments were also conducted with NMpneoT24T10 mouse mammary cells expressing the mutated c-Ha-*ras* oncogene after transfection. With this cell line, growth inhibition was enhanced when both agents were combined as was observed with the *ras*-transfected MCF-7pneo*ras*TD5 cells (Table 3).

DISCUSSION

The present experiments confirm that expression of an activated *ras* oncogene may enhance the radioresistance of cells and suggest that this enhancement can be neutralised by HePC.

The assays for growth used in the present experiments were preferred over survival curves for testing I.R. because we considered that, for the treatment of cancer, the fraction of cells recovering after growth inhibition was more important for prognosis than the fraction killed. We wanted to have 3-dimensional or at least 2-dimensional cell-to-cell contacts during as much of the experiment as possible, since this mimics the geometry of tumours more closely than single cell suspensions.

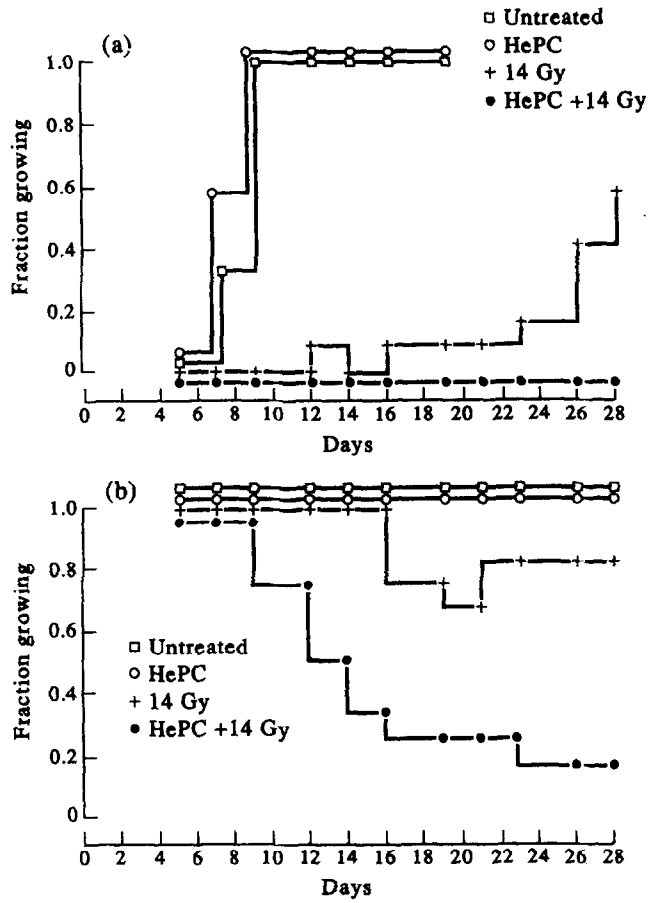

Fig. 4. Growth curves obtained with MCF-7pneo*ras*TD5 cells explanted as spheroids (panel a) or seeded as cell suspensions (panel b); cells were untreated, treated with hexadecylphosphocholine, irradiated with 14 Gy, or treated with hexadecylphosphocholine followed by irradiation. Ordinate: fraction of cultures scoring positively for growth (for criteria see Material and Methods); abscissa: time after explantation or seeding.

Table 3. Growth characteristics of spheroids treated by ionising radiation (I.R.), hexadecylphosphocholine (HePC) or a combination of both (HePC + I.R.)

Cell type (Gy; μg/ml)	Delay of growth*			Fraction growing† (at day 28)		
	I.R.	HePC	HePC + I.R.	I.R.	HePC	HePC + I.R.
MCF-7/AZ	7 (0.2)	no	10 (0.5)	1.0	1.0	1.0
(10; 10)				1.0		
MCF-7/6	5 (0.1)	−2 (0.2)	3 (0.5)		1.0	1.0
(6; 30)				1.0		
Evsa-T	3 (0.3)	no	3 (0.4)		1.0	1.0
(6; 30)				0.6		
MCF-7pneo*ras*TD5	9 (0.1)	no	> 20 (0.0)		1.0	0.0
(14; 10)	3 (0.7)	no	> 19 (0.0)	1.0	1.0	0.0
NMpneoT24T10	7 (0.1)	no	12 (0.1)	1.0	1.0	0.3
(14; 30)						

* Difference in onset of growth (i.e. area covered more than 5 mm^2) between untreated and treated cultures expressed in days; a negative value indicates an earlier onset of growth in treated as compared to untreated cultures. Figures in parentheses are the fraction of treated cultures showing growth at the onset of recovery. † With or without previous growth delay. no, no growth arrest observed. MCF-7pneo *ras* TD5: data from two sets of experiments are mentioned separately.

It has indeed been demonstrated that cell-to-cell contact greatly influences the effects of therapeutic agents [20]. In cultures seeded from single cell droplets, growth was scored earlier than in cultures seeded from spheroids because of the criteria applied. However, the final score of the fraction of cultures showing growth at the end of the assay was similar for both types of cultures, as was demonstrated in a matched experiment with MCF-7pneo*ras*TD5 cells.

The sensitivity of the present human mammary cancer cell lines to HePC was in the same order of magnitude as described for other cell lines and for other alkyllysophospholipids [1, 11, 21]. The radiosensitivity of these mammary cell lines as measured through recovery from growth inhibition was higher than that of mouse tumour cells studied earlier with similar methods of observation [22].

In our present limited series of cell types, the oncogenic *ras*-transfected and *ras*-expressing human and mouse variants were relatively radioresistant as compared with the other cell lines. This is in line with data from the literature. Expression of an activated H-*ras* oncogene did induce radiation resistance in primary rat embryo cells (RE) and this phenomenon was enhanced by the adeno E1A and by the *myc* oncogenes which by themselves did not change the response to I.R. [6–9]. With these rat cells, D_0 values increased from about 100 cGy to about 150 cGy and this 50% increase is comparable to the increase in radioresistance found with our human cell line. Radiation-induced inhibition of DNA synthesis was higher and persisted longer in the oncogene-transformed than in the parental RE cell lines, presumably through inhibition of replicon initiation [7]. Longer inhibition of DNA synthesis correlates with greater radioresistance because the delay may allow more repair and so reduce fixation of radiation damage. Radioresistance of *ras*-transfected rat rhabdomyosarcoma cells *in vitro* and *in vivo* was also ascribed to a more efficient repair of radiation-induced damage in the transfected cells [9]. Whatever its mechanisms, oncogene-induced radioresistance points to a possible genetic basis for radioresistance in conjunction with the influence of the local tumour–host microecosystem. It is worthwhile noticing that the *ras* gene product p21 constitutes one element in the signal transduction of some growth factors that stimulate DNA synthesis via AP-1 transactivation. In this pathway, PKC may be a crucial element although other protein kinases may be involved as well [4, 23, 24].

We have previously used recovery from growth inhibition in parallel with colony formation in soft agar to study radiosensitisation by chemotherapeutic agents [25]. In the present study, HePC appeared to have radiosensitising effect in the *ras*-expressing cell types, as it increased the radiosensitivity of these cells beyond the level of their non-transfected counterpart. HePC and its congeners are inhibitors of PKC both in living cells and in cell-free extracts [4, 26–28]. Other PKC inhibitors have been described as radiosensitisers and this argues in favour of a role for PKC in cellular responses to I.R. For JSQ-3 and SQ-20B cells staurosporin and sangivamycin acted as radiation sensitisers. SQ-20B cells were isolated from a squamous cell carcinoma of the larynx that had progressed after a full course of radiotherapy [29]. A possible link between the *ras* gene product, I.R. and HePC is PKC. $p21^{ras}$ transduction of signals for DNA synthesis implicate stimulation of PKC [30]. In view of this connection, HePC may counteract $p21^{ras}$ stimulation of DNA synthesis and reduce the period for potential DNA repair after I.R. Thus, $p21^{ras}$ activation may precede PKC activation and inhibition of PKC may block the *ras* signalling pathway.

To conclude, our interpretation is that *ras* makes cells more resistant to ionising radiation and that this phenomenon may be abrogated by HePC. This suggests testing of the radiosensitising activity of HePC in cancers with an activated *ras* oncogene.

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HLA Expression in Pre-invasive Cervical Neoplasia in Relation to Human Papilloma Virus Infection

Susan S. Glew, Mary E. Connor, Peter J.F. Snijders, Cynthia M. Stanbridge, C. Hilary Buckley, Jan M.M. Walboomers, Chris J.L.M. Meijer and Peter L. Stern

A significant proportion of cervical carcinomas show loss of major histocompatibility complex human leucocyte antigen (HLA) class I expression while upregulating HLA class II expression. These changes may have direct consequences for immune surveillance of the human papilloma virus (HPV) infection which is strongly associated with cervical malignancy. A relationship between changes in HLA expression and HPV infection may be evident in the evolution of premalignant disease. This immunohistological study of 104 colposcopic biopsies establishes that HLA class II expression occurs in a significant proportion of squamous epithelia showing histological evidence of wart virus infection and cervical intraepithelial neoplasia (CIN) I to III. In comparison, alteration of HLA class I expression in cervical premalignant lesions is rare. There is no correlation between the detection of high risk HPV DNA (types 16, 18, 31 and 33) by polymerase chain reaction (PCR) and the MHC class II phenotype of the lesion. This suggests that altered HLA class II expression is neither a consequence nor a prerequisite for HPV infection.

Keywords: HLA, cervical intraepithelial neoplasia, human papilloma virus, MHC class II.
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INTRODUCTION

CERVICAL CANCER and precancer form a disease continuum ranging from cervical intraepithelial neoplasia (CIN) through microinvasion to invasive carcinoma; about 70% of the tumours are squamous and 30% are adeno- and adenosquamous carcinomas [1]. Most tumours are thought to develop from an area of intra-epithelial neoplasia within the transformation zone [2]. This is at the junction of the ectocervical non-keratinising stratified squamous epithelium and the columnar epithelium lining the endocervical canal. At puberty, the increased concentration of ovarian hormones increases the bulk of the cervix leading to eversion of the columnar epithelium. Squamous metaplasia, the gradual replacement of the columnar by squamous epithelium through reserve cell proliferation [3], occurs in response to the relative acidity of the vaginal environment compared to that of the cervical canal. It is in the transformation zone that CIN may arise either by unicellular origin with horizontal spread to replace the normal epithelium [4] or by field transformation [3, 5]. An association between sexual behaviour and cervical cancer has long been observed (e.g. early age at first intercourse [6] and number of sexual partners [7]), and it is recognised that sexually transmitted infections are one of the