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Mechanisms of Cytotoxicity Induced by Horseradish Peroxidase/Indole-3-Acetic Acid Gene Therapy

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Abstract We have previously proposed the horseradish peroxidase (HRP) and the non-toxic plant hormone indole-3-acetic acid (IAA) as a novel system for gene-directed enzyme/prodrug therapy (GDEPT). The cytotoxic potential of HRP/IAA GDEPT and the induction of a bystander effect were demonstrated in vitro under normoxic as well as hypoxic tumour conditions. To date, the chemical agents and the cellular targets involved in HRP/IAA-mediated toxicity have not been identified. In the present work, some of the molecular and morphological features of the cells treated with HRP/IAA gene therapy were analysed. Human T24 bladder carcinoma cells transiently transfected with the HRP cDNA and exposed to the prodrug IAA showed chromatin condensation, formation of apoptotic bodies, DNA fragmentation, and Annexin V binding. Similar effects were observed when the cells were incubated with the apoptotic agent cisplatin. Caspases appeared to be involved as effectors in HRP/IAA-mediated apoptosis, since treatment with a general caspase inhibitor decreased the fraction of cells with micronuclei (MN) by 30%, with fragmented DNA by 50%, and with condensed chromatin by 60%. However, very little degradation of one of the downstream targets of caspase-3, PARP, could be detected, and apoptosis alone did not appear to account for the killing levels measured with a clonogenic assay. The effect of HRP/IAA treatment on cell cycle progression was also investigated, and a rapid cytostatic effect, equally affecting all phases of the division cycle, was observed. J. Cell. Biochem. 87: 221–232, 2002. © 2002 Wiley-Liss, Inc.

Key words: apoptosis; GDEPT; HRP; IAA; cancer; cell cycle

Cancer gene therapy approaches based on the delivery of genes encoding non-toxic enzymes to confer sensitivity to specific prodrugs (gene-directed enzyme/prodrug therapy, GDEPT) have been extensively and successfully adopted in pre-clinical studies and some early clinical trials [reviewed in Greco and Dachs, 2001]. We are developing a novel GDEPT system consisting of the peroxidase from horseradish (HRP) and the plant hormone indole-3-acetic acid (IAA). In vitro studies demonstrated that in HRP-transfected human cells cytotoxic prodrug activation was prompt and efficient [Greco et al., 2000, 2001]. Moreover, HRP/IAA could selectively radiosensitise tumour cells and elicit a bystander effect under oxic as well as hypoxic conditions [Greco et al., 2001, 2002]. This could represent a therapeutic advantage over well-established systems, since hypoxia is common to solid tumours and presents an adverse prognostic indicator [reviewed in Dachs and Tozer, 2000]. The HRP/IAA system has the potential to be used in a variety of anti-cancer strategies. Besides GDEPT, specific HRP-targeting to the tumour could be achieved with HRP-conjugated antibodies [ADEPT, antibody-directed enzyme/prodrug therapy; Melton and Sherwood, 1996; Folkes and Wardman, 2001] or polymers [PDEPT, polymer-directed enzyme/prodrug therapy; Connors et al., 1995; Folkes and Wardman, 2001]. IAA is well tolerated in humans [Mirsky and Diengott, 1956] and its non-specific activation in normal tissue is unlikely to take place, since mammalian peroxidases failed to convert it into a cytotoxin at

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therapeutically significant prodrug doses [Pires de Melo et al., 1997, 1998; Folkes et al., 1998].

To date, the activated drug and the cellular targets involved in HRP/IAA-mediated toxicity have not been identified. The initial hypothesis was that the IAA-peroxyl radical generated in the presence of HRP would initiate membrane lipid peroxidation [Folkes et al., 1998]. This was indeed detected in phosphatidylcholine–cholesterol liposomes incubated with HRP and IAA [Candeias et al., 1995], and found to be prevented in the presence of antioxidants such as ascorbate, Trolox, α-tocopherol, and β-carotene [Candeias et al., 1996]. However, no lipid peroxidation was detected in mammalian cells exposed to cytotoxic doses of HRP/IAA and, although Trolox was protective against cytotoxicity, probably intercepting indole radicals, preloading the cells with α-tocopherol was ineffective [Folkes et al., 1999]. On the other hand, incubation of the activated drug with plasmid DNA in a cell-free system resulted in the formation of adducts and strand breaks, indicating that DNA damage could be involved in the observed cell kill [Folkes et al., 1999]. Reactive electrophiles that are downstream products of the peroxyl radical and/or reactive carbon-centred radicals are likely to be implicated. In particular, 3-methylene-2-oxindole (MOI) has been reported to react with cellular nucleophiles such as thiols, DNA, and sulphhydryl groups in enzymes or histone [Folkes and Wardman, 2001].

Understanding the processes induced by an anticancer agent may be important not only to deduce its mechanism of action but also to improve its therapeutic efficacy and targeting potential. The aim of this work was the analysis of some of the molecular and morphological features of the dying cells treated with HRP/IAA gene therapy. The mode of cell death was examined by evaluating several characteristics of apoptosis in HRP-transfected human tumour cells exposed to IAA. The effect of drug treatment on cell cycle progression was also investigated.

MATERIALS AND METHODS

Cell Culture

Human T24 bladder carcinoma (European Collection of Cell Cultures, Salisbury, United Kingdom) and FaDu nasopharyngeal squamous carcinoma cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Paisley, United Kingdom) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma Aldrich, Gillingham, United Kingdom) and 2 mM L-glutamine (Life Technologies), in a humidified incubator at 37°C and 5% CO_2/air. Human umbilical vein endothelial cells (HUVECs) from pooled donors (TCS CellWorks, Botolph Clayton, United Kingdom) were cultured as described before [Kanthou and Tozer, 2002]. Only cells negative for mycoplasma infection (MycoTect kit, Life Technologies) were used.

Plasmid DNA and Cell Transfection

The plasmid pRK34-HRP [Connolly et al., 1994] was kindly provided by Dr. D.F. Cutler, UCL, London. The control plasmid pCMV-CD4, containing the gene for the marker protein CD4, was constructed as described previously [Dachs et al., 2000]. In both cases gene expression was driven by the cytomegalovirus (CMV) immediate early gene promoter.

Transient transfectants were obtained by exposing the cells to complexes of DNA, Lipofectin (Life Technologies), and integrin-targeted peptides [Hart et al., 1998] as described previously [Hart et al., 1998], and were assayed after 24 h.

Drug Exposure

The cells were exposed to IAA (Sigma Aldrich), cisplatin (CDDP; Platamine, Pharmacia and Upjohn, Milan, Italy), or staurosporine (Calbiochem, San Diego, CA) in phenol red free Hanks' balanced salt solution (HBSS, Life Technologies), in the 37°C incubator. The cell-permeable caspase inhibitor of broad specificity z-Val-Ala-Asp-CH_2 (zVAD.fmk) was purchased from Calbiochem.

Annexin V Binding

Cells and culture media were harvested and resuspended in binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$) at 10^6 cells/ml. The cell suspension was incubated for 15 min with 0.5 μg/ml propidium iodide (PI, Sigma Aldrich) and 5 μl Annexin V-FITC (PharMingen, San Diego, CA) at room temperature (rt), in the dark. Samples were subjected to fluorescence-activated cell sorting (FACS) analysis on a Becton Dickinson FACScan and data

were analysed using CELLQuest software for Apple Macintosh (Becton Dickinson, Franklin Lakes, NJ). Samples were gated to exclude cellular debris. Cells that showed increased FITC- or PI-related fluorescence compared to controls exposed to HBSS alone were scored as positive.

DNA Fragmentation Analysis

Following drug exposure, the cells were either assayed immediately, or the drug-containing buffer was replaced with complete media and the cells were left to recover for 24 or 48 h. To detect DNA fragmentation, cells and culture media were harvested, centrifuged, and the pellets were fixed in ice-cold 70% ethanol for 1 h. Fixed cells were resuspended in HBSS containing PI and RNase A (Sigma Aldrich) both at the final concentration of 20 μg/ml, and incubated at rt for ~30 min until analysed by FACS.

Nuclear Staining

To study nuclear integrity, chromatin condensation, and the formation of micronuclei (MN), cells were treated for different time intervals on glass coverslips. The cells were fixed in 3.7% formaldehyde (Sigma Aldrich)/PBS for 15–20 min rt, permeabilised in 0.1% Triton (Sigma Aldrich)/PBS for 5 min rt, and mounted on microscope slides in Vectashield mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA). The slides were viewed using a NIKON TE200 fluorescence microscope equipped with custom designed image acquisition software (Gray Cancer Institute, Northwood, UK). To quantify cells with condensed chromatin and MN, at least 600 cells were examined.

Detection of PARP Cleavage

Cell monolayers and any detached cells were washed in ice-cold PBS, and suspended in lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% Triton X, 5 mM EDTA, containing 10 μl/ml protease inhibitor cocktail; Sigma Aldrich). Equal quantities of proteins from such whole cell extracts were loaded into the wells of precast 6% polyacrylamide gels (Invitrogen-Novex, Groningen, The Netherlands) and subjected to SDS–polyacrylamide gel electrophoresis.

Proteins were transferred to nitrocellulose membranes (Genetic Research Instrumentation Ltd., Rayne, United Kingdom) and immunoblotting was performed with primary mouse monoclonal anti-PARP antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1,000, and secondary HRP-conjugated goat anti-mouse immunoglobulins (Dako, Ely, United Kingdom) diluted 1:2,000. Immunoreactive bands were detecting by using the enhanced chemiluminescence technique (ECL kit, Amersham Pharmacia Biotech, UK), according to manufacturer's instructions.

Cell Cycle Analysis

Prior to drug exposure, the cells were incubated for 25 min at 37°C with bromo-deoxyuridine (BrUdR; Sigma Aldrich) at the final concentration of 2 μM in culture medium. Following two washes in PBS, the cell monolayers were exposed to HBSS with or without IAA for up to 30 h. At 2 h intervals, cells and media were harvested by scraping in ice-cold PBS and centrifugation, and the pellets were resuspended in 70% ethanol.

Antibody staining was performed by incubating the samples in 0.1 mg/ml pepsin (Sigma Aldrich) in 2 M HCl for 20 min at rt, followed by mouse anti-BrUdR (Sigma Aldrich) diluted 1:25 in 0.5% normal goat serum/0.5% Tween 20 (both from Sigma Aldrich)/PBS and 1:25 FITC-conjugated goat anti-mouse immunoglobulins (Sigma Aldrich), both for 1 h at rt. The cells were resuspended in 20 μg/ml PI in HBSS for FACS analysis.

Statistical Analysis

Significance tests were carried out on the data groups by using analysis of variance (ANOVA) followed by pair-wise comparison between specific groups (Student t-test). Values of $P < 0.05$ were considered as significant.

RESULTS

Annexin V Binding

One of the early events of apoptosis is the externalisation of the phospholipid phosphatidylserine (PS), normally present on the cytoplasmic moiety of the plasma membrane [Fadok et al., 1992]. Annexin V is a protein with high affinity for PS and can bind to the surface of cells undergoing apoptosis [Schutte et al., 1998]. Using Annexin V conjugated with a fluorochrome such as FITC, apoptotic cells can be detected by using microscopy or flow cytometric procedures. Moreover, at these early stages, apoptotic cells present an intact membrane and, unless permeabilised, can exclude the DNA

fluorochrome PI. Using a non-viral delivery system [Hart et al., 1998], human T24 bladder carcinoma cells were transiently transfected with the HRP cDNA (HRP^+), and HRP-immunostaining indicated that 20–25% of the cells expressed the foreign gene (data not shown). The cells were subsequently exposed for 24 h to 0.5 or 3 mM IAA, which we previously found to induce surviving fractions of 5.9 and 0.004%, respectively [Greco et al., 2000]. In T24 cells expressing the marker CD4 ($CD4^+$), after 24 h 0.5 mM IAA was not measurably toxic and 3 mM IAA induced 57% cell kill. $CD4^+$ and HRP^+ T24 cells incubated with IAA for 24 h were assayed for Annexin V binding immediately after drug exposure. Following FACS analysis, four populations could be observed: living cells negative for both dyes, early apoptotic cells positive for Annexin V only, necrotic cells positive for PI only, and late apoptotic/necrotic cells positive for both dyes (Fig. 1). HRP^+ cells were also treated with cisplatin (CDDP; Fig. 1), previously found to induce apoptosis in T24 cells [Kawasaki et al., 1996; Ono et al., 2001].

A significant increase in Annexin V binding was selectively induced in IAA-treated HRP^+ cells, compared to buffer-treated controls (Figs. 1, 2). Induction levels (fraction of Annexin V-positive cells after IAA/fraction of Annexin V-positive cells after buffer only) of 1.9 ± 0.2 (0.5 mM IAA) and 6.1 ± 1.5 (3 mM) were measured, with average percentages of Annexin V-binding cells of 10 and 25%, respectively. Cells transfected with the gene for the marker protein CD4 did not show any increase in Annexin V binding at either prodrug concentration, while 24 h incubation with 0.7 mM CDDP induced a 5.4-fold increase in Annexin V binding (Fig. 2). Compared to HBSS-treated controls, there was a non-significant increase in PI staining in the population treated with HRP/IAA or CDDP, and the cells that were not able to exclude PI were also positive for Annexin V binding (Fig. 1). The cells were analysed after shorter incubation intervals (2 or 6 h), but no significant increase either in Annexin V binding or in PI staining could be detected (results not shown).

DNA Fragmentation

Another characteristic of cells undergoing apoptosis is the activation of endonucleases that degrade DNA. DNA cleavage could be detected by staining DNA in ethanol-fixed cells with PI followed by FACS analysis. Figure 3 shows the FACS profile of HRP^+ T24 cells exposed to buffer alone, CDDP or IAA, fixed, permeabilised, and stained with PI. Cells in the different phases of the division cycle at the time of assay can be detected. The appearance of cells with low DNA content, below that of G_1 cells (sub-G_1 peak), is considered to be a marker of cell death by apoptosis [Afanas'ev et al., 1986].

To monitor the apoptotic subdiploid population, $CD4^+$ and HRP^+ T24 cells were either assayed immediately after 24 h drug treatment, or replaced in fresh full medium for 24 or 48 h and subsequently analysed. In each experiment, the percentage of cells in the sub-G_1 peak after drug treatment relative to buffer-treated controls (relative sub-G_1 peak) was measured (Fig. 4). Immediately after drug exposure, a significant increase in apoptotic HRP^+ cells was detected at both concentrations of IAA (Fig. 4). When the cells were assayed 24 or 48 h after HRP/IAA treatment, the relative sub-G_1 peak was >1, but the increase was statistically significant only after 3 mM IAA (Fig. 4). In comparison, after 0.7 mM CDDP for 24 h, ~3-fold increase in sub-G_1 cells above buffer-treated controls was observed (results not shown). To test the involvement of caspases in HRP/IAA-induced apoptosis, the broad specificity caspase inhibitor zVAD.fmk was administered during IAA treatment. After 3 mM IAA, the cells containing fragmented DNA represented 14% of the population immediately after exposure, 58% at 24 h and 62% at 48 h. In the presence of 100 μM zVAD.fmk these percentages were reduced by about 50%, suggesting the involvement of caspases in the process (results not shown).

Chromatin Condensation

Due to rapid dehydration, apoptotic cells often become diminished in size, or elongated and convoluted in shape. Chromatin condensation and the loss of distinct chromatin structure occur in parallel with cell shrinkage, and they are usually followed by nuclear fragmentation [Wyllie, 1992]. Nuclear fragments, together with the constituents of the cytoplasm, are then packaged into apoptotic bodies, which enveloped in plasma membrane, detach from the dying cell. The nuclear morphology of HRP^+ T24 cells exposed to 3 mM IAA for varying lengths of time (2, 4, 6, 8, 14, 18, and 24 h) was monitored by fixation and staining with

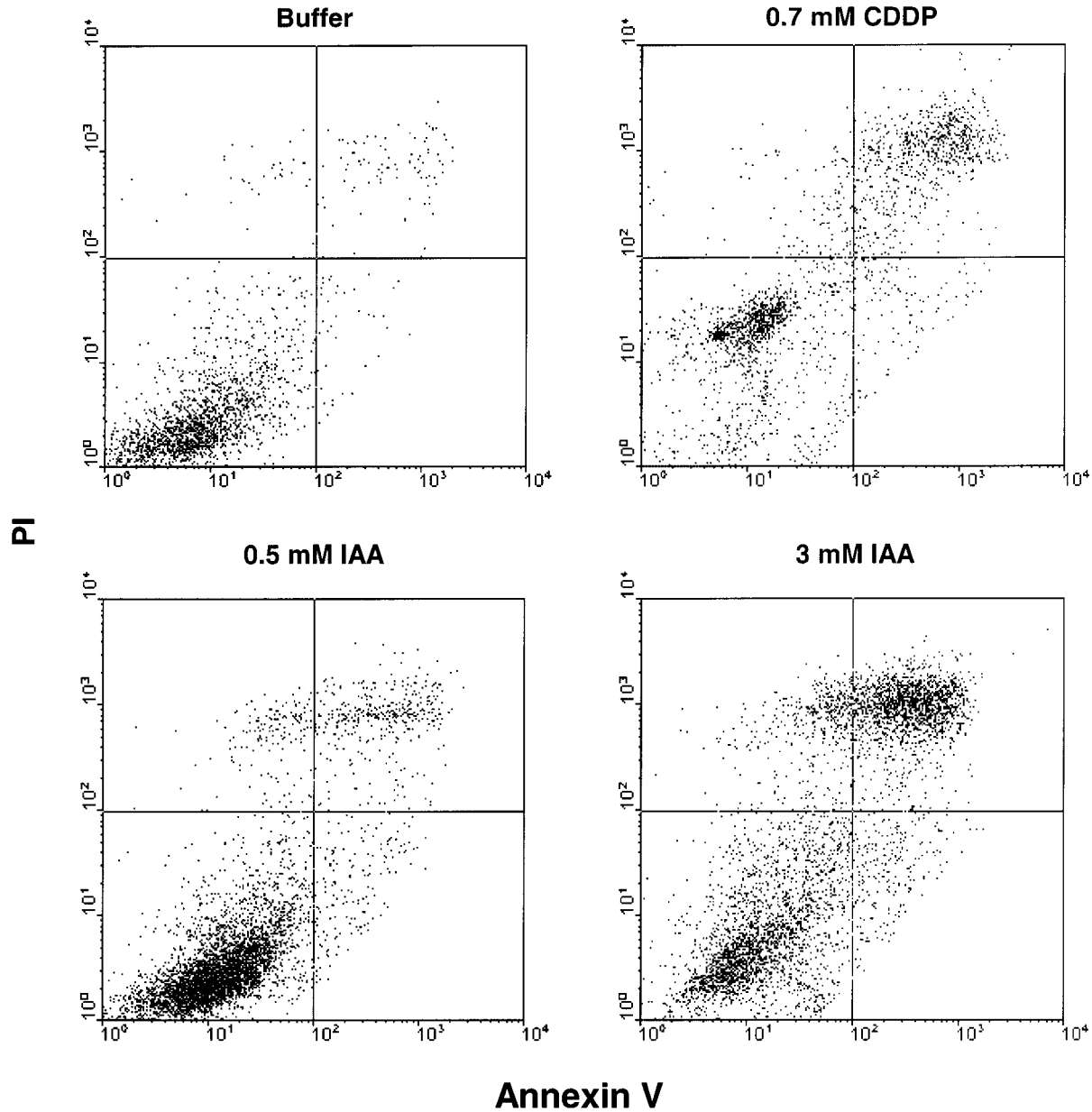

Fig. 1. Analysis of Annexin V binding in T24 cells. T24 cells transiently transfected with the horseradish peroxidase cDNA (HRP^+) were exposed for 24 h to buffer, 0.7 mM cisplatin (CDDP), or indole-3-acetic acid (IAA) at the doses of 0.5 or 3 mM. As a measure of apoptosis, the cells were assayed for Annexin V binding by FACS immediately after drug exposure. Propidium iodide (PI) staining indicates loss of cell membrane integrity.

DAPI (Fig. 5 shows the effects at 24 h). From 4 h on, fluorescence microscopy revealed the presence of condensed chromatin, especially at the nuclear periphery, characteristic of apoptosis. Some of the cells treated with HRP/IAA presented highly damaged, shrunken, dehydrated nuclei (Fig. 5E). Similar damaged nuclei have been observed in T24 cells after co-administration of vitamins C and K_3 [Gilloteaux et al., 2001].

Condensed chromatin and shrunken nuclei were also observed in T24 cells exposed to CDDP (Fig. 5B), whereas no major changes were observed in the nuclei of HRP^+ cells exposed to HBSS (Fig. 5A) or CD4-transfectants exposed to IAA (data not shown).

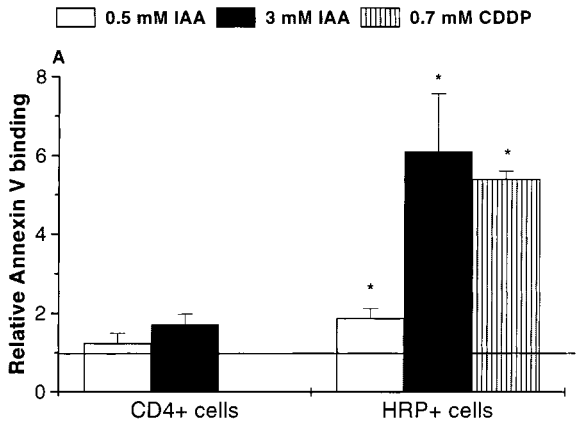

Fig. 2. Annexin V binding of T24 cells treated with HRP/IAA gene therapy. $CD4^+$ or HRP^+ cells were exposed for 24 h to 0.7 mM CDDP, 0.5 or 3 mM IAA. Relative Annexin V binding, assayed as in Figure 1, was expressed as the percentage of positive cells after IAA/percentage of positive cells after buffer only. The means of three independent experiments ± SE are indicated. *Significantly different from 1 ($P < 0.05$).

Cells with condensed chromatin were counted and normalised to the total number of cells in the observation field. At least 600 cells were examined. After 3 mM IAA for 24 h, HRP^+ T24 cells with condensed chromatin represented 3.9% of the exposed population. This percentage was reduced to 1.5% in the presence of 100 μM zVAD.fmk. The caspase inhibitor had no effect on HRP^+ cells exposed to buffer or $CD4^+$ cells incubated with IAA (results not shown).

Cells treated with HRP/IAA were also characterised by the presence of micronuclear structures, probably due to nuclear fragmentation, and to the formation of apoptotic bodies (Fig. 5F). Compared to HBSS-treated controls, the frequency of MN in HRP^+ cells was markedly increased after 6 h-exposure to 3 mM IAA (Fig. 6). After 24 h, 0.109 (± 0.008) MN/cell were detected in HRP/IAA-treated cells, against a baseline of 0.039 (± 0.008) MN/cell in untreated $CD4^+$ cells. zVAD.fmk inhibited the formation of MN, reducing their frequency to baseline levels (0.041 ± 0.005 MN/cell after 24 h 3 mM IAA and 100 μM zVAD.fmk).

Condensed chromatin, MN, and damaged nuclei were also selectively induced in HRP^+ human nasopharyngeal squamous carcinoma FaDu cells exposed to 3 mM IAA (data not shown).

PARP Degradation

When the apoptotic process is initiated, one of the downstream events of caspase-3 activation is the cleavage of the DNA repair enzyme Poly (ADP-ribose) polymerase (PARP) into two inactive subunits of 27 and 85 kDa [Pieper et al., 1999]. In the last few years, PARP degradation has been used extensively as a marker of apoptosis [Kaufmann, 1989].

PARP cleavage was monitored in HRP^+ T24 cells incubated with 3 mM IAA for up to 24 h by Western blotting. Only very little cleavage of PARP could be detected in the HRP/IAA-treated samples, as well as in cells exposed to 0.7 mM CDDP (Fig. 7 shows a typical experiment). HUVECs exposed for 12 h to 0.2 μM staurosporine were used as a positive control (Fig. 7).

The lack of PARP cleavage after HRP/IAA treatment was not limited to T24 cells, as it was also observed in the FaDu cell line (data not shown).

Cell Cycle Analysis

In order to evaluate the effect of HRP/IAA treatment on cell cycle progression, HRP^+ and $CD4^+$ T24 cells were pulse-labelled with BrUdR and subsequently exposed to HBSS or 3 mM IAA for up to 30 h. This observation interval was chosen to monitor a division cycle, since analysis of the growth of cultured T24 cells indicated a doubling time of ~24 h (data not shown). Cell samples were collected every 2 h, and FACS-analysed after antibody and PI staining.

While cells exposed to buffer only showed a normal cell cycle progression, HRP^+ cells incubated with IAA did not appear to progress through the cycle for the entire observation time (Fig. 8). This cytostatic effect was confirmed by the quantification of the fraction of labelled cells in G_1 (Fig. 9).

The results were specifically dependent on the activated drug, as $CD4^+$ cells exposed to the same dose of IAA were not affected by prodrug treatment (data not shown).

DISCUSSION

HRP and IAA represent a novel combination for GDEPT of cancer. The chemical and biological pathways leading from prodrug activation to cytotoxicity are yet to be elucidated. The experiments presented here were carried out in order to shed light on the death mechanisms induced in human tumour cells.

Several pathways leading to apoptosis have been studied. The apoptotic machinery can be broadly divided into "sensors" and "effectors".

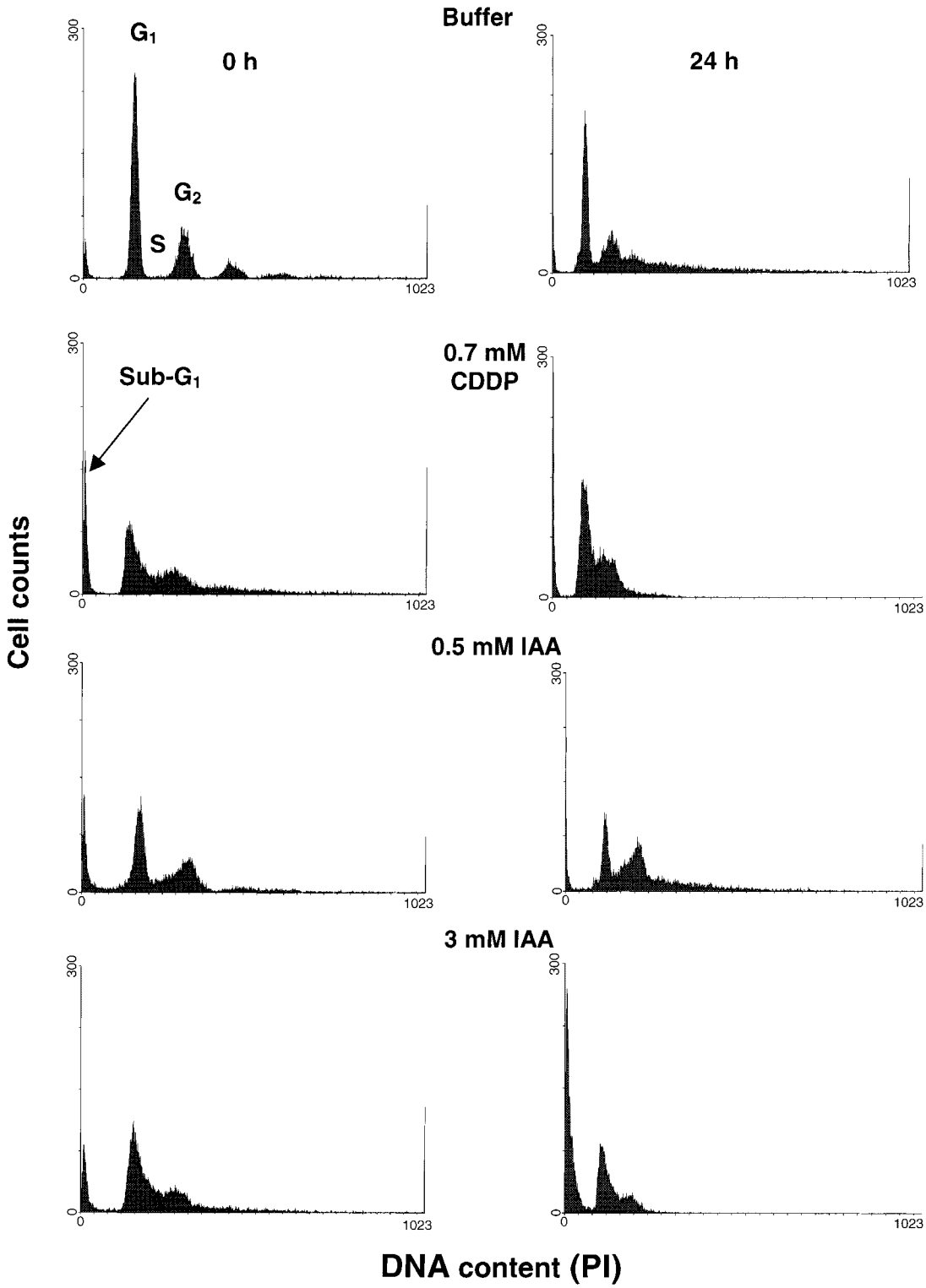

Fig. 3. DNA fragmentation analysis in T24 cells by FACS. HRP^+ T24 cells exposed to buffer only, 0.7 mM CDDP, 0.5 or 3 mM IAA were fixed and stained with PI and analysed by FACS. In the experiments shown here, the cells were incubated with the drugs for 24 h and assayed immediately after. G_1, S, G_2, and sub-G_1 cells are shown.

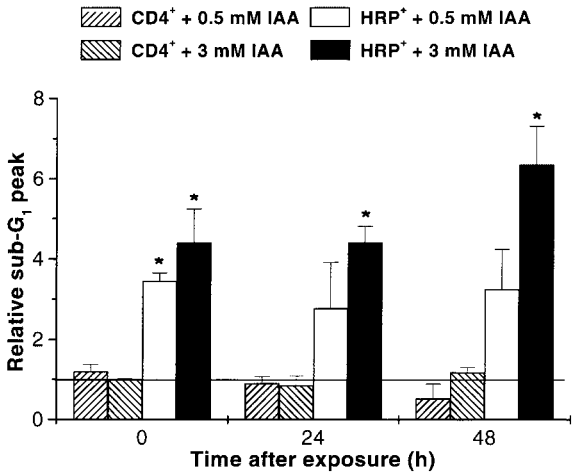

Fig. 4. HRP/IAA-induced DNA fragmentation. HRP^+ and $CD4^+$ T24 cells were exposed for 24 h to 0.5 or 3 mM IAA, and the percentage of cells in the sub-G_1 peak after IAA relative to buffer-treated controls (relative sub-G_1 peak) was assessed either immediately (0 h), or after a 24 or 48 h recovery period. The means of at least three independent experiments and the SEs are indicated. *Significantly different from 1 ($P < 0.05$).

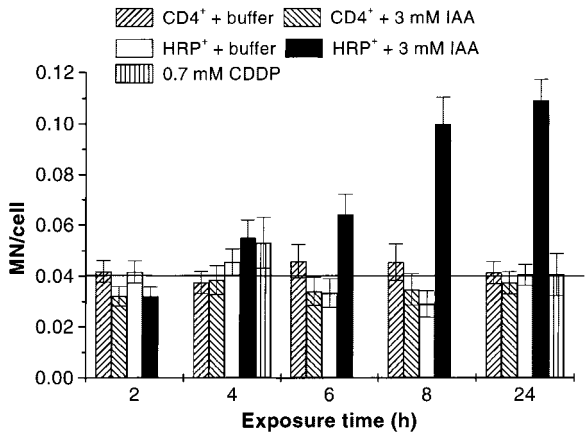

Fig. 6. Quantification of micronuclei (MN). At different time intervals during drug exposure, T24 cells were fixed, the nuclei were stained with DAPI and visualised by fluorescent microscopy. At least 600 cells were examined per time point and MN were scored. The data represent the average number of MN per total number of cells. The base line indicates the number of MN/cell in untreated $CD4^+$ cells.

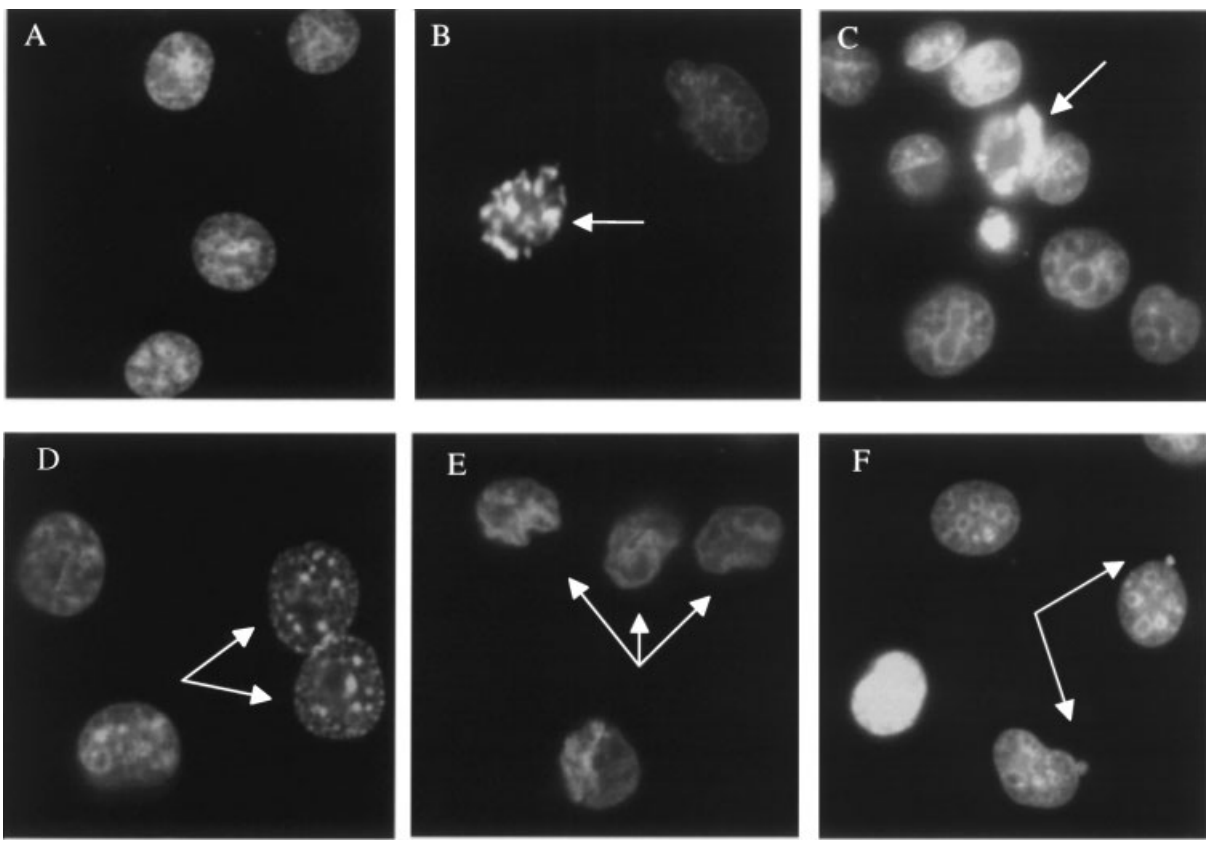

Fig. 5. Chromatin condensation, damaged nuclei, and apoptotic bodies in T24 cells. After drug treatment, nuclei were fixed, stained with DAPI, and viewed using a NIKON TE200 fluorescence microscope (100 × objective). Representative images are shown. **A**: HRP^+ cells, no drug. **B**: HRP^+ cells + 0.7 mM cisplatin (CDDP), 24 h. **C–F**: HRP^+ cells + 3 mM IAA, 24 h. Arrows indicate nuclei with condensed chromatin (B–D), damaged, shrunken nuclei (E), and apoptotic bodies (F).

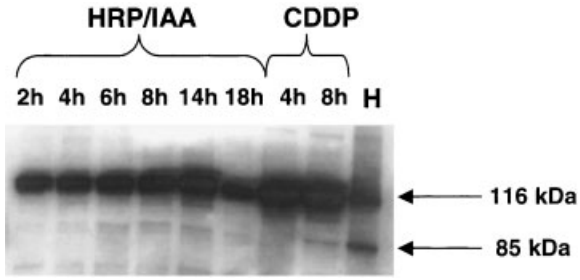

Fig. 7. Cleavage of PARP. HRP^+ T24 cells were exposed to 3 mM IAA and cell extracts were analysed by Western blotting after 2, 6, 8, 14, and 18 h. Alternatively, the cells were incubated with 0.7 mM CDDP for 4 or 8 h. HUVECs treated with 0.2 μM staurosporine were used as positive controls (H).

Sentinels such as cell surface receptors that bind to survival or death factors (e.g., FAS ligand, TNF-α) are responsible for monitoring the extracellular environment for conditions that affect the decision of the cell to live or die. Intracellular sensors monitor abnormalities including DNA damage, oncogene activation, survival factor deficiency, or hypoxia. For instance, the p53 tumour suppressor protein, a key inducer of the apoptotic cascade, can elicit apoptosis by upregulating expression of *Bax* in response to sensing DNA damage [reviewed by Hanahan and Weinberg, 2000]. In turn proteins of the Bcl-2 family, whose members have either pro-apoptotic (Bax, Bak, Bid, Bim) or antiapoptotic (Bcl-2, Bcl-XL, Bcl-W) function, act in part by governing mitochondrial death signalling through release of cytochrome *c*, a potent catalyst of apoptosis [Hanahan and Weinberg, 2000]. Among the ultimate effectors of apoptosis are an array of intracellular proteases, including the set of caspases, which can activate other caspases or execute the death programme through selective degradation of subcellular structures, organelles, and DNA.

Identification of apoptotic cells is undoubtedly complex, and some of the features associated with apoptosis may also accompany cell necrosis [Collins et al., 1992]. Therefore, apoptosis may be identified with a higher degree of assurance only by using more than one assay.

Overall our results indicate that an apoptotic pathway may be activated by HRP/IAA gene therapy. Similar to the apoptotic agent CDDP, HRP/IAA selectively induced exposure of phosphatidylserine, DNA fragmentation, and chromatin condensation in the treated cells (Figs. 1–6). Caspases appear to be involved as effectors, since treatment with the general caspase inhibitor zVAD.fmk decreased the fraction of cells with MN by 30%, with fragmented DNA by 50%, and with condensed chromatin by 60%. On the other hand, a functional p53 appears not to be required, since T24 and FaDu cells both express mutated forms of this protein [Reiss et al., 1992; Kawasaki et al., 1996].

However, very little degradation of one of the downstream targets of caspase-3, PARP, could be detected. The DNA repair enzyme PARP is selectively activated by DNA strand breaks to catalyse the addition of long branched chains of poly (ADP-ribose) from its substrate nicotinamide adenine dinucleotide [NAD; Pieper et al., 1999]. Proteolytic cleavage of PARP may occur when the cell is no longer able to repair or replicate its DNA and initiates the disassembly that ensures its commitment to apoptosis. However, the complete role of PARP in the apoptotic process remains to be determined, since $PARP^{-/-}$ cells display different levels of susceptibility towards apoptosis compared to their wild type counterparts [Pieper et al., 1999]. Here, very little PARP cleavage could be detected in FaDu and T24 cells treated with HRP/IAA as well as CDDP (Fig. 7). This was, unexpected as CDDP has been shown to induce apoptosis by a caspase 3-dependent pathway [Cenni et al., 2001], and degradation of PARP has been observed in *p53* mutants [Hanai et al., 2001]. Specific data on PARP cleavage in T24 and FaDu cells could not be found in the literature, but there is a growing body of evidence that in many cell systems apoptosis may be atypical, lacking one or more of the features that characterise classical apoptosis [Cohen et al., 1992].

Cells that initiate apoptosis cannot be clonogenic, yet the percentages of apoptotic cells detected after HRP/IAA GDEPT cannot fully account for the killing levels measured with a clonogenic assay [Greco et al., 2000, 2001]. For example, 0.5 mM IAA induced up to 30% sub-G_1 and up to 10% Annexin V-positive cells in the HRP^+ population, while clonogenicity revealed 94% cell kill after the same prodrug dose. Indeed PI staining of unfixed cells suggested that some cells underwent necrotic death, although the increase was not significant and restricted to the Annexin V-positive population (Fig. 1). More experiments may be needed to assess the role and kinetics of necrotic death in the exposed population. Several investigators have

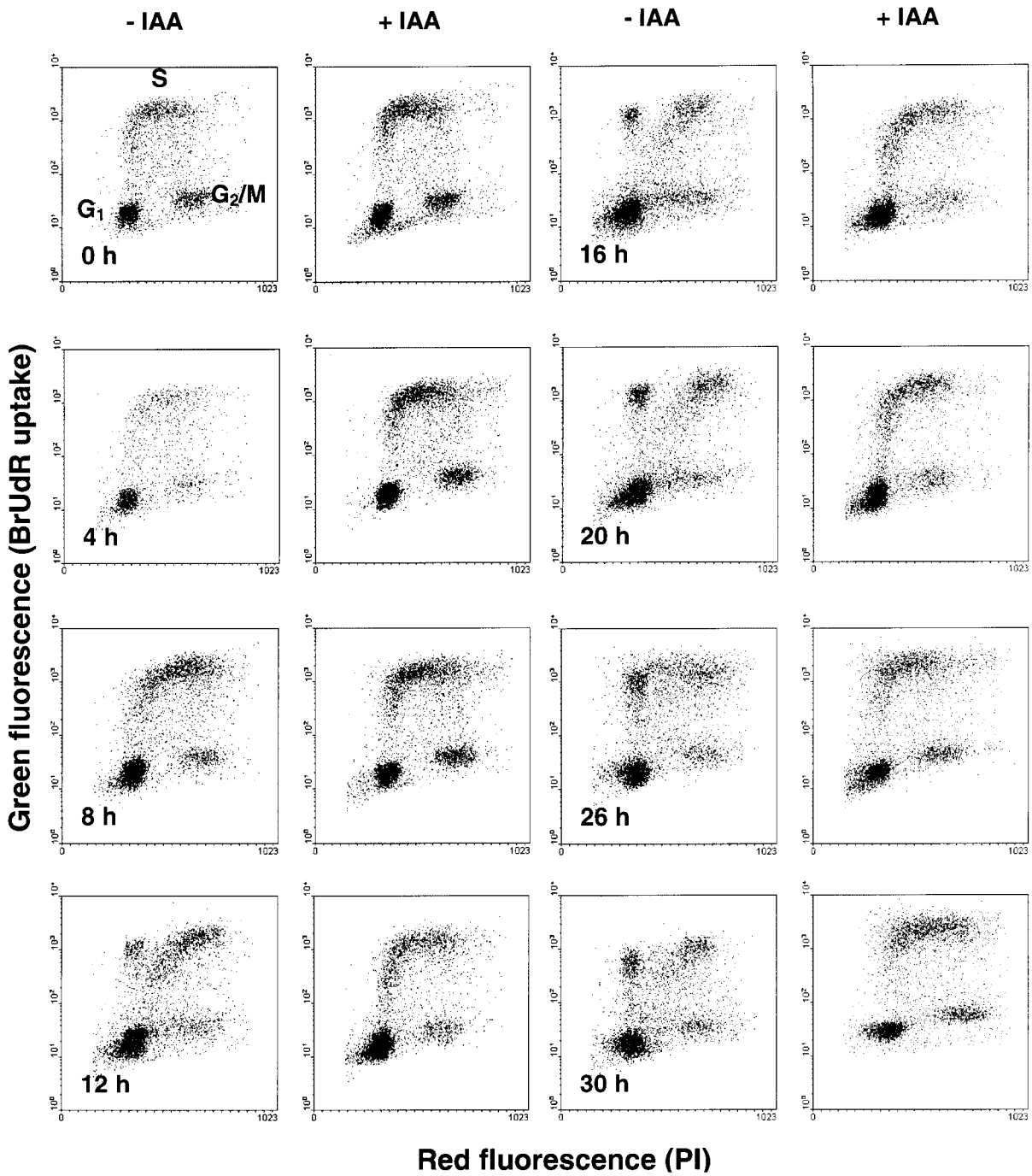

Fig. 8. Cell cycle analysis of HRP/IAA-treated T24 cells. HRP^+ cells were pulse-labelled with BrUdR, exposed to 3 mM IAA (+ IAA) or buffer only (− IAA) for up to 30 h and FACS-analysed at 2 h intervals. Selected profiles of a representative experiment are shown. The data were displayed as two-parameter cytograms. Three subpopulations, representing G_1 cells (low green and low red fluorescence), G_2/M cells (low green and high red), and S-phase cells (high green), are indicated.

observed that cell sensitivity as assessed by clonogenic ability is greater than that measured by apoptosis assays [reviewed in Brown and Wouters, 1999]. The relationship between apoptosis and clonogenic survival is complex, and they may or may not be correlated. Moreover, apoptosis is a dynamic process and could be underestimated by examining cells at a certain time point. In particular, at the time of detection the cells appeared not to have

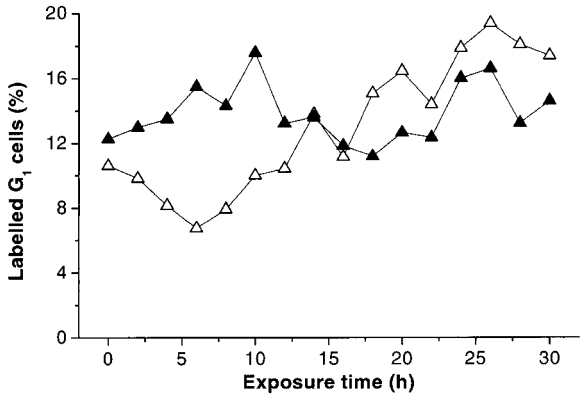

Fig. 9. Cell cycle progression of T24 cells treated with HRP/IAA. At 2 h intervals for 30 h, T24 cells were treated, and analysed as in Figure 8 and the BrUdR-positive cells in the G_1 phase were counted and normalised to the total number of cells assayed. The fraction of labelled cells in G_1 in one representative experiment is shown. HRP^+ T24 cells exposed to IAA (▲) or buffer only (△).

attempted to undergo mitosis yet, as suggested by the cell cycle data (Figs. 8, 9). Delayed apoptosis [particularly important for *p53*-mutated cells; Brown and Wouters, 1999] may, therefore, be occurring.

Indeed, the signal to the cells to die could arise from their inability to progress through the cell cycle. HRP/IAA gene therapy caused a rapid cytostatic effect, equally affecting all phases of the division cycle (Figs. 8, 9). Interestingly, in accordance with the clonogenic assays, almost the entire exposed population was affected, even though only ~20% expressed the HRP and were, therefore, able to activate IAA. This observation correlates with the survival data from the clonogenic assays that point towards a significant bystander effect of the HRP/IAA combination [Greco et al., 2000, 2001].

The initiating event triggering the apoptotic response is yet to be identified. Experiments carried out with the purified enzyme indicated binding and accumulation of [5-^{3}H] IAA in the cell nucleus [Folkes and Wardman, 2001]. Moreover, DNA adducts and strand breaks were observed in plasmid DNA incubated with activated IAA [Folkes et al., 1999], and extensive DNA fragmentation was revealed with pulsed field gel electrophoresis in T24 cells (own unpublished data).

The results presented here may help in understanding the mode of action of HRP/IAA-mediated toxicity, which could lead to improvements in GDEPT, ADEPT as well as PDEPT approaches. Further work is necessary to fully elucidate the initial damage and the pathways involved, and the impact of the observed apoptosis on tumour eradication will need to be assessed in in vivo models.

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