Mechanisms of Resistance of Confluent Human and Rat Colon Cancer Cells to Anthracyclines: Alteration of Drug Passive Diffusion

Hélène Pelletier, Jean-Marc Millot, Bruno Chauffert, Michel Manfait, Philippe Genne, and François Martin

Research Group on Digestive Tumors, INSERM U252, Faculty of Medicine, 7 Bd Jeanne d’Arc, 21033 Dijon Cedex [H. P., B. C., P. G., F. M.], and Laboratory of Biomolecular Spectroscopy, Faculty of Pharmacy, 51 rue Cognacq Jay, 51096 Reims Cedex [J. M. M., M. M.], France

ABSTRACT

Two colon cancer cell lines, HT-29 (human) and DHD/K12/TRb (rat), were grown as monolayer cultures to various confluence degrees. The cytotoxic efficacies of doxorubicin and 4’-deoxydoxorubicin, evaluated by a survival assay, and the nuclear drug concentrations, measured by microspectrofluorometry, were shown to progressively decrease with the augmentation of confluence. This confluence dependent resistance (CDR) to anthracyclines was demonstrated independent of the multidrug resistance drug efflux mechanism. The cellular uptake of three compounds (sodium [*Cr]*Crchromate, D-l[*C]*alanine, l-[14C]glucose) known to passively diffuse across the cell membrane as anthracyclines do was also reduced in confluent cells. After trypsin cell detachment, the kinetics of reversion of the sodium [*Cr]*Crchromate uptake decrease and that of CDR were similar. Therefore, CDR may be attributed to a reduction of anthracycline cell intake due to a general alteration of passive diffusion across the cell membrane. However, CDR is only partly explained by this phenomenon since a reduced sensitivity of confluent cells was observed compared with nonconfluent cells for a similar amount of drug in their nuclei. CDR could explain the high resistance to anthracyclines of some solid tumors, such as colon tumors, in which cancer cells are tightly aggregated.

INTRODUCTION

Anthracyclines are among the most active anticancer agents, widely used in treatment of solid tumors and leukemias. However, a natural or acquired anthracycline resistance of tumors often hinders the curative potentiality of these drugs.

One of the most investigated mechanisms of anthracycline resistance consists of an increased active drug efflux out of the cancer cell supporting, at least partly, their MDR phenotype (1–3). Verapamil (4), amiodarone (5), and some other compounds are able to inhibit this efflux and to circumvent anthracyclines resistance in some experimental cancer cell systems.

However, in solid tumors other mechanisms than MDR have been put forward to explain their resistance to anthracyclines: alteration of vascularization; low drug penetration through multiple cell layers, as clearly demonstrated by experiments on spheroids; and deleterious effect of low pH and pO2 in tumor microenvironment (6). In the present study, we have showed a dramatic diminution of nuclear accumulation and cytotoxicity of anthracyclines related to the increase of the cellular confluence of monolayer cultures of two colon cancer cell lines. Our results have led us to suggest that the increase of confluence induces a general alteration of passive diffusion across the cell membrane and, as a consequence, a reduction of the cell intake of anthracyclines.

MATERIALS AND METHODS

Drugs. DXR was obtained from Roger Bellon Laboratories (Neuilly-sur-Seine, France). deoDXR was a gift from Farmitalia Laboratories (Milan, Italy). Amiodarone was obtained from Labaz Laboratories (Bordeaux, France) and was used in this study for inhibiting the active anthracycline efflux transport in PROb cells (5). Stock solutions (1 g/l) of these drugs were prepared in distilled water. Final dilutions were prepared in Ham’s F-10 medium immediately before each experiment.

Cell Lines. The HT-29 cell line of human colon adenocarcinoma was originally established from a xenograft by Fogh and Trempe (14). The DHD/K12/TRb cell line (referred to as PROb) was established in our laboratory from a transplantable colon adenocarcinoma induced by 1,2-dimethylhydrazine in syngeneic BD IX rats (15). PROb cells exhibit a primary resistance to anthracyclines due partly to an active drug efflux (16). By immunoblotting, using MRK-16 antibody, we have shown the existence of membrane P-180 glycoproteins in PROb cells but their absence in HT-29 cells (data not published). These two cell lines were maintained as monolayers in tissue culture flasks using Ham's F-10 medium supplemented with 10% fetal bovine serum. Cells were detached by trypsin digestion with EDTA and trypsin.

Survival Assay. To determine the cytotoxic effect of anthracyclines a previously described colorimetric test was used (17). Briefly, tumor cells were cultured for a constant time of 48 h in microtiter 96-well flat-bottomed plates and the different stages of confluence were obtained by the variation of inoculum (nonconfluence, 10⁶ cells/well; subconfluence, 5 x 10⁵ cells/well; confluence, 15 x 10⁵ cells/well). In the case of HT-29 cells, it was possible to obtain an additional stage of hyperconfluence after 8 days of culture (15 x 10⁵ cells/well) without cell detachment. Then, cells were treated with drugs for 1 h. After rinsing, cells were cultured for 72 h in complete medium. In some experiments, tumor cells at different stages of confluence in culture flasks were detached by trypsin and suspended for 1 h in anthracycline solutions at a same drug:cell ratio (5 x 10⁶ cells/ml) whatever was the previous confluence status of the cells. After washing, cells were seeded (10⁶ cells/well) in microtiter 96-well flat-bottomed plates and cultured for 72 h in complete medium. In all cases, after the posttreatment incubation, cells were rinsed twice in PBS in order to remove nonadherent dead cells. Then, cells were fixed for 10 min in absolute ethyl alcohol and stained with 1% methylene blue in 0.01 M borate buffer, pH 8.5. After abundant rinsings, the dye bound to residual cells was eluted with 0.1 N hydrochloric acid and its absorbance was measured on an automatic photometer (Multiskan, Flow Laboratories, Irvine, United Kingdom) equipped with a 630 nm filter. Absorbance of the eluted dye was demonstrated to be proportional to the number of residual target cells. Each determination issued from a quadruplicate. Results were expressed as

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\text{Cell survival} = \frac{\text{Mean absorbance in treated wells}}{\text{Mean absorbance in control wells}} \times 100
\]

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: MDR, multidrug resistance; DXR, doxorubicin; deoDXR, 4’-deoxydoxorubicin; CDR, confluence dependent resistance; PBS, phosphate buffered saline.
Microspectrofluorometer. Fluorescence emission spectra from an intracellular microvolume were recorded with a prototype microspectrofluorometer obtained from a modified OMARS 89 Raman Spectrometer (Dilor, Lille, France) as already described (18, 19). By means of a conventional optical microscope (Olympus BH2) equipped with a x100 water phase contrast immersion objective (Olympus), a laser beam was focused on a spot less than 1 μm in diameter. Sample observation and collection of fluorescence emission were obtained through the same optics. The actual fluorescence sampling was restricted by means of a suitable pinhole diaphragm on the image plane of the microscope objective. The emission light signal, spectrally dispersed with a diffraction grating, was detected with an optical multichannel analyzer, made of a cooled 512-diode array, optically coupled to an image intensifier. Data were collected locally and then transferred to a Goupi G4 computer for analysis with a specifically developed program (18). At least 20 spectra from the same intracellular location were accumulated in order to obtain a signal/noise ratio of about 30. A Rhodamine B solution was used as a fluorescence standard in order to control laser power and instrumental response day by day and to permit quantitative comparisons between spectra recorded on different days. Sample heating, photobleaching, and photodamage were checked empirically and found to be negligible under our experimental conditions: a 4-μW laser power on the sample; and an illumination time of 0.5–1 s. In particular, cells always remained viable after repeated fluorescence determinations, as checked by phase contrast microscopy.

Determination of Nuclear Anthracyclines Concentrations in Living Cells. For anthracyclines uptake studies, cells cultured on glass coverslides were incubated in Ham’s F-10 medium containing the appropriate drug concentration for 1 h. Then, they were washed free of drug in cooled PBS at 4°C and the glass cover slide was placed in a Petri dish containing PBS. The fluorescence emission arising from the nucleus of a cell treated with DXR or deoDXR can be expressed as a sum of the spectral contributions of free drug, DNA-bound drug and intranuclear auto-fluorescence. By studies in aqueous solutions, we showed in a recent paper (18), that each of these contributions has a characteristic spectral shape and that the fluorescence yield in the free form is 48 times higher than that of the DNA-bound form. Thus, the drug concentration in the living cell nucleus was obtained from the determined surface spectral contributions and by means of the corresponding fluorescence yields (18). Intranuclear concentrations of drugs were expressed in μmolar.

Radioactive Molecule Cell Uptake Measurements. Cells were trypsinized and adjusted to 5 × 10⁵ cells/ml. In a tube, 200 μl of the cell suspension were mixed either with 70 μl of a Na⁵¹CrO₄ solution (1 mCi/ml; CEA, Gif/Yvette, France) or 40 μl of a D-[¹⁴C]glucolone solution (50 μCi/ml; Amersham, Buckinghamshire, England) or 50 μl of a L-[¹⁴C]glucolone solution (200 μCi/ml; Amersham). After a 1-h incubation at 37°C, cells were washed and adjusted to 10⁶ cells/ml. The radioactivity of aliquots of 100 μl of the cell suspensions was measured through a gamma counter (LKB 1272 Clini Gamma, Stockholm, Sweden) or through a beta counter (LKB 1214 Rackbeta). Each result was the mean of eight determinations.

RESULTS

Sensitivity to Anthracyclines of HT-29 and PROb Cells Treated at Different Degrees of Confluence. The cell densities corresponding to the different degrees of confluence of both HT-29 (human) and PROb (rat) colon cancer cell monolayers cultures were: nonconfluence, 309 ± 94 cells/mm²; subconfluence, 1805 ± 206 cells/mm²; confluence, 3478 ± 627 cells/mm²; hyperconfluence, 7000 ± 851 cells/mm². A general observation was that sensitivity of HT-29 and PROb cells to DXR and deoDXR progressively decreased with the augmentation of cell confluence (Fig. 1). The different cell densities from nonconfluence to confluence were obtained by different inocula of cancer cells cultured for a constant time. In that way, we could ascertain that this resistance was independent of the duration of culture but only linked to confluence. Thus, in the course of the following study, we often worked with two extreme cell densities of nonconfluence and hyperconfluence in HT-29 cells, this latter cell density being obtained after 8 days of culture.

Since a reduction in glucose or oxygen supply or a low pH were described as causes of anthracycline resistance (12, 20) we checked that similar results were obtained when medium was renewed every 12 h during the 48 h before treatment (data not shown).

Both cell lines were more sensitive to deoDXR than to DXR, but the confluence effect was potent with both anthracyclines. However, the cytotoxicity of deoDXR only clearly decreased with the higher level of confluence obtained with each cell line. Thus, the importance of anthracycline resistance arising with confluence appeared to be modulated by the efficacy of the cytotoxic drug.

It was previously demonstrated that PROb cells resist anthracyclines in part through an active drug efflux pump (5) identified as a p180 glycoprotein whereas HT-29 cells did not possess this mechanism of resistance. When PROb cells were treated in presence of amiodarone, an inhibitor of this pump, the cytotoxic effects of DXR and of deoDXR were increased (Fig. 2). A maximal effect of amiodarone was obtained at a dose of 5 μg/ml. However, even in presence of amiodarone, the confluence effect on PROb cell sensitivity to DXR and deoDXR persisted. For HT-29 cells, amiodarone had no effect on the cytotoxicity of the two anthracyclines at any stage of cell confluence (data not shown). Thus, the anthracycline resistance occurring with the augmentation of confluence of the two cell lines appeared independent of an active drug efflux mechanism since this phenomenon was observed when the efflux was inhibited or did not exist. We have further used the term CDR for confluence dependent resistance. In all the subsequent experiments, we treated PROb cells in the presence of amiodarone whereas HT-29 cells were treated in the absence of this efflux inhibitor.

After trypsinization, the CDR of hyperconfluent HT-29 cells to anthracyclines was stable at least up to 6 h but had disappeared within 24 h (Fig. 3).

Nuclear Concentrations of Anthracyclines in HT-29 and PROb Cells Treated at Different Degrees of Confluence. The nuclear accumulation of DXR or deoDXR progressively decreased with the augmentation of confluence for both HT-29 cells and PROb
cells (Fig. 4). In the particular case of PROb cells, treated with 10 μg/ml of deoDXR in presence of amiodarone, the nuclear drug concentrations were not significantly different between nonconfluent and confluent cells, suggesting that saturation of DNA was reached under these conditions of treatment. The cytotoxicity under these conditions was actually complete for both nonconfluent and confluent PROb cells (Fig. 2).

We observed a high discrepancy between the different stages of confluence concerning the relation between the level of anthracycline nuclear accumulation and the cytotoxicity induced. For example, for HT-29 cells, DXR at a dose of 1 μg/ml led to a nuclear accumulation of about 50 μM and a cell mortality of 50% for nonconfluent cells whereas for hyperconfluent cells, treated with 10 μg/ml DXR, a nuclear accumulation of 230 μM induced a cell mortality of only 5%. Thus, a nearly 5-fold higher DXR nuclear accumulation in hyperconfluent HT-29 cells than in nonconfluent ones induced a smaller cytotoxic effect. These results clearly demonstrated that CDR to anthracyclines had two causes: (a) a decreased accumulation of drug in the nucleus of confluent cells; and (b) a nuclear resistance of the confluent cell to the cytotoxic effect of the drug.

For PROb cells, similar ratios of nuclear anthracycline concentrations in nonconfluent cells to those in confluent cells were obtained whether they were treated in presence of amiodarone or not (Table 1). These results confirmed that CDR was independent of an active drug efflux mechanism.

We wondered whether anthracycline influx in confluent cells could be altered by the reduction of cell surface in contact with the drug and/or by a reduced drug:cell ratio. When HT-29 cells of different confluence degrees were trypsinized adjusted to similar cell densities, and immediately treated with anthracyclines in similar conditions, it still appeared that former hyperconfluent cells were more resistant to anthracyclines than former nonconfluent cells (Fig. 5A). We observed for deoDXR, but not for DXR, that HT-29 cells treated in suspension had an altered capacity to adhere again during the survival assay. Thus, we had to reduce the deoDXR concentrations in the treatment of cells in suspension, as compared to that of cells in monolayer. The nuclear concentrations of DXR or deoDXR in former nonconfluent cells were higher than in former hyperconfluent cells (Fig. 5B) as observed with cell monolayers (Fig. 4). Thus, the reduced nuclear anthracycline accumulation in confluent cells may likely be related to a change of the confluent cell compared with the nonconfluent one.

Passive Diffusion Analysis. It is now admitted that anthracyclines passively diffuse across the cell membrane as neutral compounds (21). We studied the confluence effect on the cell uptake of three radioactive molecules known to cross the membrane independently of active transport mechanisms. These molecules were Na235CrO4 and D-[14C]alanine and L-[14C]glucose, the two isomers of the natural forms of these components. The passive diffusion of these molecules was checked by linear
RESISTANCE TO ANTHRACYCLINES OF CONFLUENT COLON CANCER CELLS

Fig. 4. DXR and deoDXR uptake in the nucleus of HT-29 and PROb cells treated (1 h, 37°C) at different degrees of confluence: nonconfluent (□), confluent (○), hyperconfluent (▲). Drug concentration in the nucleus was quantified by microspectrofluorometry.

Table 1 Nuclear concentrations (μM) of DXR in nonconfluent (NC) and confluent (C) PROb cells treated for 1 h in the presence or not of amiodarone

<table>
<thead>
<tr>
<th>Cell confluence</th>
<th>Amiodarone (5 μg/ml)</th>
<th>DXR doses (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>−</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>−</td>
<td>10</td>
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* Nuclear drug concentrations were determined by microspectrofluorometry immediately after treatment.

Fig. 7. DXR and deoDXR uptake in the nucleus of HT-29 and PROb cells treated (1 h, 37°C) at different degrees of confluence: nonconfluent (□), confluent (○), hyperconfluent (▲). Drug concentration in the nucleus was quantified by microspectrofluorometry.

DISCUSSION

Using two colon cancer cell lines, cultured as monolayers, this study clearly established a progressive development of resistance to anthracyclines when confluence increased. We partly related this phenomenon to a decreased accumulation of anthracyclines in the nucleus as measured at this site by microspectrofluorometry, a recently developed method (18).

The best-known mechanism of resistance to anthracyclines in cancer cells is an active drug efflux pump preventing anthracyclines from accumulating in their nuclei and conferring on them, at least partly, the multidrug resistance (MDR) phenotype (1–3). The use of an inhibitor of this efflux pump, amiodarone (5), did not suppress the confluence dependent resistance (CDR) and demonstrated that this phenomenon was independent of the MDR mechanism. We supposed that the reduced drug accumulation in nuclei of confluent cells could be due to an alteration of the anthracycline uptake by cells. It could not be attributed to differences in drug:cell ratio among nonconfluent, confluent, and hyperconfluent monolayers which could induce differences in effective drug concentrations delivered per cell. Indeed, when nonconfluent or hyperconfluent HT-29 cells were trypsinized and immediately treated in suspension at a same drug:cell ratio, the same difference of nuclear drug accumulation was observed as when cells were treated as monolayers. In the same way, the reduction of DXR or deoDXR uptake on account of a decreased membrane surface in contact with the drug in confluent cells was excluded by our results since the detachment by trypsin of hyperconfluent HT-29 cells before treatment did not reverse either their low drug nuclear accumulation or their resistance. These results demonstrated that the decreased anthracycline nuclear accumulation in confluent cells was not artifactual but linked to a modification of the confluent cell compared with the nonconfluent one. Because anthracyclines passively diffuse across the cell membrane (21),

[Graphs and tables are included here for visual representation of data and results.]
we checked the effect of confluence on the intake of nonmetabolizable molecules known to penetrate into the cell by passive diffusion because of the absence of a specific transport mechanism (sodium [14C]chromate) or of the stereospecificity of existing transport mechanisms (D-[14C]alanine; L-[14C]glucose). Since these three molecules were less uptaken by confluent than by nonconfluent HT-29 cells, we concluded that parameters generally conditioning passive diffusion were altered with confluence. Consequently, a reduced passive diffusion of anthracyclines across the membranes of confluent cells could explain the low nuclear drug accumulation in these cells and thus, at least partly, the CDR. The observation that the confluence related decrease in Na[51Cr]O4 uptake and that the anthracycline CDR had strictly similar kinetics of reversion, reinforced a possible link between the level of sensitivity to anthracyclines.
of the cell and the general level of passive diffusion across its membrane. Experiments to determine whether confluence induced modifications could be related to membrane fluidity and thus to membrane composition are in progress.

However, the reduction of anthracycline passive diffusion and the low nuclear accumulation of these drugs in confluent cells cannot entirely explain CDR. Indeed, we observed that, for a similar nuclear anthracycline accumulation, confluent cells appeared to be more resistant than nonconfluent cells. Thus, confluence appeared to induce nuclear changes in addition to the membrane modifications suggested above.

Confluence, and hyperconfluence even more, corresponds to the stationary phase of growth of PROb and HT-29 cells. A decreased efficacy of anthracyclines on tumor cells in a stationary phase of growth compared with exponentially growing cells has been widely reported for monolayer cultures (8–12) as well as for suspension cultures (22). The most common assumption in these previous studies to explain the resistance of stationary phase cells was their noncycling state. For 4′-(acridinylamino)methanesulfon-m-anisidine, another intercalating agent, it was recently demonstrated that the resistance of noncycling cells could lie in an alteration of the drug-DNA topoisomerase II interaction (23, 24). In cultured human cells an important decrease of the intracellular DNA topoisomerase II level when cell density increased was demonstrated and primarily related to G2-G1 (25). In our study, 83% of confluent and 69% of nonconfluent HT-29 cells were accumulated in G2-G1 as observed by flow cytometry (data not shown). This difference in cell cycle repartition was significant even weak. Whether a membrane modification and/or a defect of the nuclear target of anthracyclines (DNA topoisomerase II) could be linked to this increase of cells in G2-G1 phase has to be checked. However, a 1-h exposure to 10 μg/ml of DXR or deoDXR killed more than 80% of nonconfluent HT-29 cells, suggesting that these drugs could be cytotoxic even for cells in G2-G1 if they were nonconfluent. At the same concentration, the two anthracyclines killed no more than 20% of hyperconfluent HT-29 cells. Moreover, microspectrofluorometry experiments revealed that the difference of anthracycline nuclear accumulation between the different cell densities concerned the totality of the cell population and not a subpopulation as expected if it was proliferation dependent. Indeed, as an example, we observed for nonconfluent and hyperconfluent HT-29 cells a correct homogeneity of measurements (from 30 cells) inasmuch as the standard deviations were, respectively, 11 and 22% of the mean level of deoDXR nuclear concentrations. Thus, these results prevent us to strictly relate the CDR to a lack of cell cycle progression.

As a conclusion, the anthracycline CDR phenomenon, demonstrated in this work from two colon cancer lines in vitro, could be of first importance to explain the high resistance of colon tumors to these drugs. Indeed, CDR could be the main factor leading cancer cells to resist anthracyclines when they are tightly aggregated as in solid tumor.

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