Abstract. In solid tumors, chemotherapeutics must adequately diffuse through the extracellular compartment to achieve their cytotoxic effect. Using quantitative microspectrofluorometry, both Doxorubicin penetration through three-dimensional (3D) collagen I matrices and its subsequent intranuclear accumulation into HT-1080 cells cultured in this microenvironment were directly assessed. Evidence that collagen delayed the Doxorubicin penetration for 1 h is presented. During that period, drug concentrations were lower in the nuclei in 3D compared to 2D matrices. Anthracyclines were also found to exhibit similar cytotoxicity in 2D and 3D after long term incubation. In conclusion, in this 3D culture model, collagen type I matrices delayed the early distribution of low molecular weight (mw) therapeutics and failed to affect their long-term cytotoxic effects, as previously reported. This model may provide a rationale for avoiding the emergence of intrinsic chemoresistance in tissue. The cell microenvironment appears to be a key determinant in the emergence of de novo multidrug resistance, a major obstacle to the successful use of antitumor drugs. Based on the direct contact between cancer cells and the extracellular matrix or adjacent cells, these resistance mechanisms have been described to involve decreased proliferation and apoptosis, alterations in drug target and in integrin-mediated signaling (1). However, such studies were performed using multicellular spheroids or tumor cells coated onto matrix proteins. They did not concern cells maintained in three-dimensional (3D) matrices allowing cells surrounded by the substrata to develop new types of cell/matrix interactions – recently referred to as 3D-matrix adhesions (2). These adhesions differ in structure, location and function compared to classic 2D-adhesions, thus, making them relevant models that closely mimic in vivo conditions. In addition, impaired delivery of drugs to tumor cells may play a role in de novo chemoresistance (3). Indeed, the fibrillar collagen network could affect the diffusion of low molecular weight (mw) therapeutics into 3D matrices as was shown for macromolecules (4).

Whether a 3D microenvironment may modify both cellular uptake and cytotoxic effect of Doxorubicin, a low mw anthracycline, was investigated here. Doxorubicin ranks among the most active anticancer agents, widely used in treatment of leukemias and solid tumors. This drug, in order to reach its main intracellular targets, DNA or topoisomerases, has to be transported across the plasma membrane via a flip-flop mechanism (5), supporting a prominent role for the cell membrane in determining anthracycline chemosensitivity. As a fluorescent molecule, Doxorubicin levels can be quantified at the single living cell level using a microspectrofluorimetric approach (6). In this report, both its diffusion in a 3D collagen I matrix and its intranuclear uptake in single human fibrosarcoma HT-1080 cells cultured in this matrix were quantified. The effects of this microenvironment on the cytotoxic activity of Doxorubicin and Aclacinomycin, another structurally-related anthracycline currently used clinically, were also evaluated.

Materials and Methods

Cell culture and drugs. HT-1080 cells (CCL-121) were purchased from the American Type Culture Collection (Rockville, MD, USA) and were maintained as monolayers under standard culture conditions. The anthracycline drugs were Doxorubicin (TEVA Pharma, Puteaux, France) and Aclacinomycin (Sigma, Isle d’Abeau Chesnes, France).

Collagen gels. Acid-extracted, non-pepsinized collagen I from rat tail tendons was prepared as already described (7). Lyophilized collagen I was dissolved in sterile 0.1% acetic acid (3 mg/mL). After trypsinization and washes with PBS, £10^5 cells were resuspended in 100 µL fetal calf serum (FCS) and were mixed with a solution containing 100 µL MEM 10X, 100 µL NaHCO₃ 0.26 M, 110 µL H₂O, 90 µL NaOH 0.1 M and 500 µL collagen 3 mg/mL.
This solution was deposited in a 35-mm plastic dish (Nunc, Roskilde, Denmark) and after gelling at 37°C for 10 min, it was recovered by 1 mL MEM 10% FCS. Collagen gels without cells were also prepared.

Confocal laser microspectrofluorometry. Fluorescence emission spectra from microvolumes of collagen matrices (drug diffusion) or of cell nuclei (drug uptake) were recorded with a microspectrofluorometer (M51, Jobin-Yvon / Horiba, Longjumeau, France) coupled with an argon ion laser (Spectra Physics, Évry, France).

Cell growth inhibition. For 3D cultures, collagen gels containing cells were seeded in 24-well plates (500 µL/well). After gelling, they were covered by 1 mL MEM 10% FCS during 24 h. The culture medium was replaced with fresh medium containing the appropriate dilutions of drugs. After 72 h, the gels were digested by collagenase 5 UI/mL (Roche, Meylan, France) and cell viability and number were determined by phase contrast microscopy. For 2D cultures, a similar procedure was followed without collagen and collagenase treatment.

Results and Discussion

The overall 3D architecture of a collagen type I gel (1.5 mg/mL) obtained by confocal reflection imaging is shown in Figure 1A. It presents a highly fibrillar organization made of 3D oriented and interconnected fibers, as reported previously in gels of comparable concentration (8). The nature of the collagen used is a telopeptide-intact collagen I. Telopeptides correspond to the flanking regions of collagen molecules; they form intra- and intermolecular crosslinks that promote the staggering and the resilience of fibrillar collagen (9).

Doxorubicin diffusion in this type of collagen gel prepared from 0.5 and 1.5% w/v solutions was analyzed by quantitative microspectrofluorometry (Figure 1B and C). For this, gels were overlayed with Doxorubicin 10^{-6} M and the time-course of drug concentrations from collagen microvolumes (1 µm^3) was determined at various collagen thickness levels at 37°C. For collagen 1.5% (Figure 1B), after only 15 min incubation, Doxorubicin incorporation into the collagen gel was severely limited for the different depths tested (5, 200 and 400 µm). Indeed, Doxorubicin concentrations at 200- and 400-µm depth only represented 56 to 48%, respectively, of the initial concentration in the above culture medium. Depending on incubation time, drug concentrations progressively increased and reached 97% at 60 min. This inhibition of diffusion also depended on collagen content, since the diffusion rate increased with decreasing collagen concentration. As shown in Figure 1C, for a lower collagen concentration (0.5%), diffusion was more rapid since drug concentrations ranged from 71 to 66% after 15 min. These data indicate that at physiologically relevant concentrations (0.5-1.5%) (8), collagen poses a significant barrier to the diffusion of small molecules, such as Doxorubicin.
as Doxorubicin (mw 580). The data are also consistent with those recently obtained in tumors in which higher diffusional hindrance has been demonstrated with higher levels of collagen type I organized into fibrils (4). However, our findings were surprising since hindrance has been previously observed with macromolecules, such as IgG, dextran or bovine serum albumin, but not for low mw compounds (10). As recently suggested for macromolecules, unassembled collagen present in the void spaces of fibrillar collagen may play a role in such a transport hindrance (8).

To provide direct information regarding drug uptake in tumor cells in a 3D environment, experiments were performed in the presence of human HT-1080 fibrosarcoma cells cultured in 3D collagen. As shown in Figure 2A, cells cultured for 24 h in collagen I expressed a markedly different geometry from that of cells cultured onto 2D surfaces. With plastic or collagen-coated surfaces, cells developed a typical teardrop shape with a wide leading edge that extended in the front and a narrow tail. In contrast, in 3D collagen, the cells presented an extremely elongated morphology concomitant

Figure 2. A) Typical morphologies of human fibrosarcoma HT-1080 cells cultured on plastic (left) or into 3D type I collagen gel (right). Note that cells on plastic adopt the classic teardrop shape with a wide leading edge (see arrowhead). In contrast, cells in 3D collagen gel are able to form a spindle shape morphology (see arrow). Bar, 40 µm. B) Comparison between 2D and 3D cultures, of Doxorubicin uptake into the nucleus of single HT-1080 cells depending on incubation time. Results are the mean values of three different experiments and each experimental point represents an average of 20 independent cell measurements. *p<0.05.
of an activated cell phenotype since they continue to proliferate in this microenvironment (36 vs. 24 h of doubling time in 3D and 2D, respectively). Similar morphological changes have been described for fibroblasts plated onto cell-derived 3D matrices and not with 2D matrix components or 3D matrices mechanically compressed (2). Using microspectrofluorometry, Doxorubicin nuclear concentration was directly examined in single HT-1080 embedded in collagen overlayed with Doxorubicin 10⁻⁶ M and treated for 1-18 h. Doxorubicin fluorescence emission spectra of this pharmacologically relevant concentration (11) were collected from microvolumes within living cell nuclei and results were expressed in terms of intranuclear Doxorubicin concentration (6). At 1 h, a significantly less important concentration occurred in the 3D-cells (Figure 2B), which can be interpreted as the consequence of Doxorubicin diffusion hindrance into the gel as reported above. Intranuclear Doxorubicin concentration progressively increased with incubation time to reach a plateau at 8 h either in 2D- or 3D-cells (Figure 2B). For longer incubations (4, 8 and 18 h), drug accumulation was similar in both populations.

In addition, intranuclear accumulation into collagen gel of lower concentration (0.5 mg/mL) was similar to that of 1.5 mg/mL (data not shown), suggesting that collagen present in the void spaces was still sufficient for drug hindrance. An estimation of drug uptake in collagen concentrations of 0.25 and 0.125 mg/mL was impossible to be performed due to gel retraction induced by the tumor cells. Finally, the sensitivity of 3D-cells to Doxorubicin and Aclacinomycin anthracyclines, was compared to that of cells on plastic (Table I). Both drugs exhibited comparable growth-inhibitory effects in 2D- and 3D-cells, with even more pronounced effects for Aclacinomycin 10 nM in 3D. These data strongly contrast previous data implicating the tumor cell microenvironment as a particularly important determinant in the emergence of chemoresistance (1). Consequently, our model may provide a rationale to prevent the resistance to the cytotoxic effect of antitumor drugs. Future identification of 3D- vs. 2D-dependent signaling events could allow for an elucidation of the mechanisms involved.

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