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Short communication

Expression of verapamil hypersensitivity in multidrug-resistant cells grown as multicellular spheroids

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Summary. It is known that certain multidrug-resistant cell lines are hypersensitive to verapamil and to some other membrane-active agents. We examined the expression of verapamil hypersensitivity in a multidrug-resistant, verapamil-hypersensitive CHO cell line by cells grown in the form of multicellular spheroids. We found that although vincristine resistance is expressed at high levels in multicellular spheroids, we could not detect verapamil hypersensitivity when cells were grown in this form. Using a dry autoradiographic method to study the entry of [^{3}H]-verapamil into the spheroids, we demonstrated that the lack of hypersensitivity was not due to incomplete drug penetration. We therefore propose that when hypersensitive multidrug-resistant cells are grown as three-dimensional spheroids, cellular interactions may modify the expression of verapamil hypersensitivity.

Introduction

Multidrug resistance in cultured cells can be partially reversed by the presence of verapamil or certain other membrane agents. Studies on this phenomenon may contribute to our understanding of multidrug resistance, and it has been suggested that the combination of verapamil, or functionally similar drugs with lesser side effects, with anticancer drugs may be of clinical value in overcoming the serious problem of drug resistance [8, 13, 16].

The reversal of multidrug resistance is probably due to the inhibition of antitumour drug efflux from resistant cells [6–8, 17], which possibly involves competition for sites involved in drug efflux between the anticancer drug and the resistance modifier. It has been shown that verapamil and other calcium channel blockers inhibit the ATP-dependent high-affinity vincristine binding to plasma membranes of resistant cell lines [9] and has been suggested that both anticancer drugs involved in multidrug resistance and resistance modifiers such as verapamil bind to the P-glycoprotein competitively and are actively transported from the cell by a common mechanism [9].

Although the general observation is that verapamil partially reverses multidrug resistance, it has also been observed that some multidrug-resistant cell lines are hypersensitive to verapamil and membrane-active agents when present alone (i.e. in the absence of an anticancer drug) [2, 4, 19, 20]. This observation may provide some indication of the cellular changes involved in the development of multidrug resistance and may be of significance during the clinical use of combinations of anticancer drugs with verapamil or related agents if the phenomenon occurs in vivo. We have been studying two multidrug-resistant/verapamil-hypersensitive Chinese hamster ovary (CHO) cell lines that have LD_{10} values of around 0.3 μg/ml verapamil as opposed to an LD_{10} value of around 30 μg/ml for unselected CHO cells [21]. For comparison, it may be noted that plasma levels of around 4 μg/ml may be achieved clinically, although these levels lead to unacceptable cardiac toxicity [13]; levels of 1–2 μg/ml may, however, be acceptable [14]. These two CHO cell lines are hypersensitive to other membrane-active agents, posses elevated levels of the membrane P-glycoprotein and of two low-molecular-weight cytosolic proteins (mol. wt., 27,000; pI, 6.1 and 6.4) and accumulate less verapamil than do control cells [21].

Growth of cells as three-dimensional multicellular spheroids provides a model that simulates micrometastases or intervascular microregions of larger tumours and is of intermediate complexity between two-dimensional monolayer cultures in vitro and tumours in vivo [11, 15]. It has previously been shown that vincristine resistance is expressed in spheroids [22]. To begin to consider the possible clinical relevance of the phenomenon of verapamil hypersensitivity in multidrug-resistant cell lines, we examined the response of one such cell line, VCR/T, to verapamil by cells grown as multicellular spheroids rather than as monolayer cultures (used in our previous experiments).

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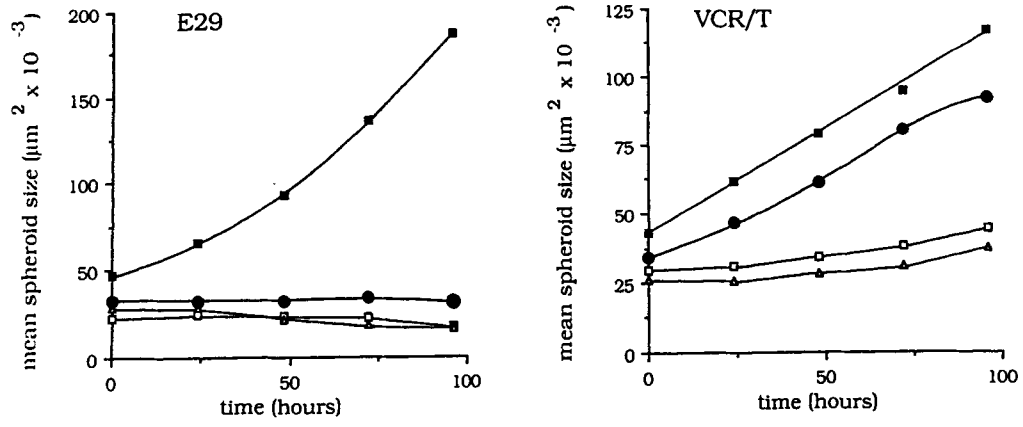

Fig. 1. Growth of E29 and VCR/T multicellular spheroids after 72 h exposure to vincristine. ■, untreated; ●, 1 μg/ml vincristine; □, 10 μg/ml vincristine; △, 100 μg/ml vincristine. The SEM was in all cases <10,000 μm² and, in the case of drug-treated spheroids, in all cases <5,000 μm²

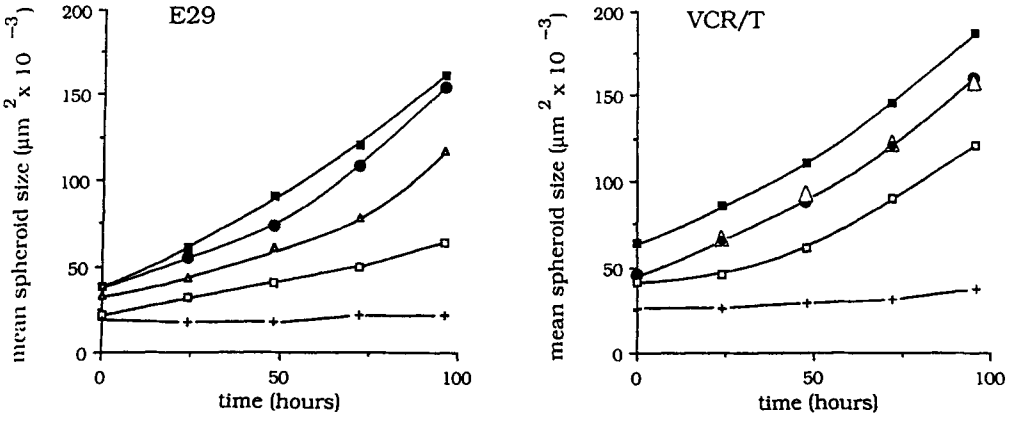

Fig. 2. Growth of E29 and VCR/T multicellular spheroids after 72 h exposure to verapamil. ■, untreated; ●, 10 μg/ml verapamil; △, 25 μg/ml verapamil; □, 50 μg/ml verapamil; +, 100 μg/ml verapamil. The SEM was in all cases <9,000 μm². The growth curves for VCR/T cells in 10 and 25 μg/ml verapamil were so closely similar that a single line was drawn through both sets of points

Materials and methods

VCR/T is a multidrug-resistant CHO cell line selected by 15 rounds of subculture in progressively increasing concentrations of vincristine [20] from an adenosine-requiring auxotropic CHO cell line that was originally obtained from V. Ling, Ontario Cancer Institute. Its properties have previously been described [21].

Cells were grown in Glasgow Minimum Essential Medium (MEM) (Gibco) with 10% foetal calf serum, supplemented with 10 mg/l adenosine, at 37° C in an atmosphere containing 5% CO_2. Multicellular spheroid cultures were initiated by adding around 10^6 cells to 10 ml medium in a 25-cm² cell-culture flask, the base of which had been previously coated with 4 ml medium solidified with 1% Difco purified agar to prevent cell adhesion. After 24 h, the liquid contents of the flask were added to a further 10 ml medium in an 80-cm² flask coated with 10 ml medium solidified with 1% agar. After 1 or 2 days, small spheroids had begun to form and the medium, which contained a mixture of single cells and small spheroids, was transferred to a conical-based universal tube in which the spheroids were allowed to settle out. The medium containing single cells was aspirated off; the remaining spheroids were transferred to 500 ml medium in a 500-ml Techne spinner flask and incubated for 3–5 days with stirring at 20 rpm. Thereafter, 20 ml spheroid stock suspension culture was transferred to an agar-coated flask (80 cm²), with the appropriate drug concentration in the liquid and solidified media. After 72 h, spheroids with sizes of 10,00-80,000 μm² (corresponding to diameters of 113–319 μm) as measured using a 40/10 image analyser (Analytical Measuring Systems, Ltd., Saffron Waldon, UK) were selected and transferred individually to wells of a 24-multiwell dish using a Pasteur pipette. Each well was coated with 300 μl solidified medium, with 2 ml liquid medium above. The size of individual spheroids was then measured at 24-h intervals. At least 20 spheroids were taken at each drug concentration and for each cell line. Experiments were carried out on two separate occasions, with similar results. Vincristine was a gift of E. Lilly and verapamil hydrochloride was purchased from Sigma.

Penetration of [³H]-verapamil into spheroids was studied using the dry autoradiographic method of Nederman [10–12], with very slight modifications. Spheroids were allowed to attach to coverslips in 100 mm petri dishes for 7 h before being incubated for 20 h in medium containing 0.24 MBq/ml (methyl-[³H])-verapamil (NEN Dupont) and an appropriate concentration of non-radioactive verapamil. Spheroids were quickly frozen at −185° C in a liquid propane-isopentane mix and then freeze-dried at −70° C. They were then vapour-fixed in a paraformic aldehyde-saturated atmosphere at 80° C before being wax-embedded using xylene. After careful removal of the coverslips, 7-μm sections were cut and dry-mounted onto microscope slides and an autoradiograph was prepared by placing the slide with the section in contact with a slide coated with Ilford K5 photographic emulsion. The two slides were tightly clipped together for 4 weeks before being developed using Kodak D19.

Results

We first examined whether the VCR/T cell line expressed its vincristine resistance when cells were grown as multicellular spheroids. The outgrowth of VCR/T and E29 spheroids following exposure to vincristine is shown in Fig. 1. The slightly slower growth rate of the untreated control spheroids of the VCR/T vs E29 cell line is to be expected, as VCR/T has a slower growth rate than E29 when grown as a monolayer culture. The two cell lines have doubling times of 18 and 14 h, respectively.

The two cell lines showed different responses to vincristine. The E29 spheroids were reduced in size after the 72-h period of exposure to 1, 10 or 100 μg/ml vincristine and showed no growth during the subsequent 96 h in

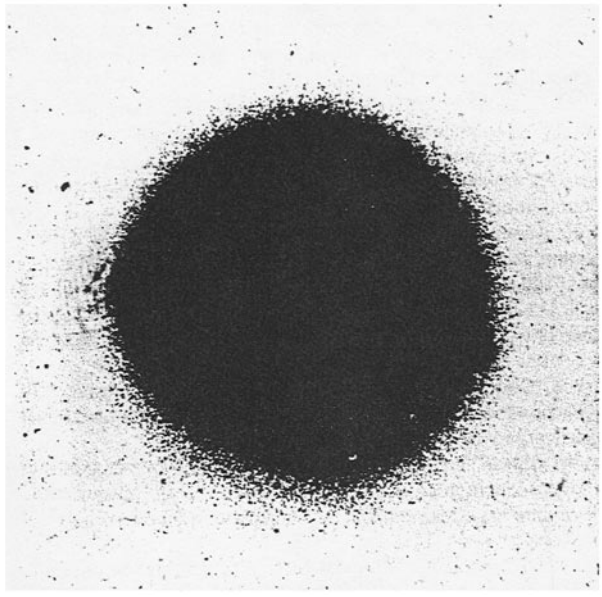

Fig. 3. Autoradiograph of a VCR/T spheroid following 20 h incubation in the presence of [^{3}H]-verapamil, demonstrating penetration of [^{3}H]-verapamil throughout the centre of the spheroid. × 250

drug-free medium. Those which had been exposed to 100 μg/ml vincristine showed a statistically significant (5% level, *t*-test) decline in size after 72 or 96 h in drug-free medium. The VCR/T spheroids were also smaller than control spheroids after exposure to these vincristine concentrations, but those that had been exposed to 1 μg/ml subsequently grew at a rate similar to that of control spheroids when transferred to drug-free medium. Some growth of VCR/T spheroids after treatment with 10 and even 100 μg/ml vincristine was also observed (significant at 1% after 72 h and at 0.1% after 96 h in 10 μg/ml vincristine, and significant at 0.5% after 96 h in 100 μg/ml vincristine). The growth curves after exposure to these two concentrations presumably reflect the outgrowth from a minority of cells surviving in the spheroid after treatment with vincristine. Overall, the data show that VCR/T, which was selected for vincristine resistance whilst being grown as a monolayer, also expresses vincristine resistance when grown as multicellular spheroids and that this resistance can be detected over a wide range of drug concentrations. The very different experimental techniques used in spheroid and monolayer culture preclude precise quantitative comparison of the expression of VCR/T resistance under the two growth conditions, but they are of comparable order. In monolayers the LD_{10} value of VCR/T cells has been reported to be 66-fold that of control cells [21], and in the present study, resistance could be detected over a 100-fold range of vincristine concentrations in spheroids.

Secondly, we examined whether VCR/T expressed verapamil hypersensitivity when cells were grown as multicellular spheroids. The outgrowth of VCR/T and E29 spheroids following exposure to verapamil is shown in Fig. 2. The duration of exposure and the verapamil concentrations used were chosen following preliminary experiments with E29 that defined the range of treatments producing a response ranging from no inhibition to complete inhibition of outgrowth. Exposure to 5 μg/ml verapamil resulted in no reduction in outgrowth as compared with untreated spheroids in both VCR/T and E29 (data not shown). Exposure to 10, 25 and 50 μg/ml resulted in partial inhibition of outgrowth (Fig. 2), which was comparable for the two cell lines. Exposure to 100 μg/ml verapamil completely inhibited outgrowth of both cell lines for 3 days. A very slight increase in the size of VCR/T spheroids was observed after 4 days, but this was not statistically significant. There appears to be no evidence for detectable verapamil hypersensitivity of the VCR/T vs E29 cell line when the cells are grown under these conditions.

The observation that VCR/T does not shown detectable verapamil hypersensitivity when grown as multicellular spheroids could reflect a physiological difference in the response of VCR/T cells to verapamil when they are grown as multicellular spheroids rather than in monolayer culture, or it could be due to the failure of verapamil to penetrate to all of the cells in the multicellular spheroid. We therefore examined the penetration of [^{3}H]-verapamil into VCR/T and E29 spheroids using the dry autoradiographic technique of Nederman (10–12). Following a 20-h exposure of VCR/T and E29 spheroids to [^{3}H]-verapamil in medium containing 10, 25, 50 or 100 μg/ml verapamil, we found that verapamil penetrated completely to the centre of all spheroids of both cell lines and at all concentrations studied. An example of an autoradiograph obtained by exposure of a VCR/T spheroid grown in 10 μg/ml verapamil is shown in Fig. 3.

Discussion

We showed that a multidrug-resistant (MDR) cell line that shows very great hypersensitivity to verapamil when grown as a monolayer culture does not express a significant level of verapamil hypersensitivity when grown as multicellular spheroids, although vincristine resistance is still expressed. It is possible that by chance we failed to detect a very slight degree of sensitivity to verapamil detectable in VCR/T cells at a level of verapamil that is intermediate between the concentrations used in our experiments. However, we feel that this is unlikely, as we did observe partial inhibition of both of these cell lines at some concentrations of verapamil. Our observations that vincristine resistance is expressed in multicellular spheroids and that higher drug concentrations are required to kill both resistant and control cells grown as spheroids rather than as monolayers are both in agreement with similar findings by Wibe [22].

Insofar as the multicellular spheroid system has some similarities to a solid tumour, our observation that verapamil hypersensitivity is not expressed in the former implies that it may not be expressed in the latter. Verapamil has been found to increase the vincristine sensitivity of human tumour tissue [6]; when this observation is taken in conjunction with our current finding, it may indirectly suggest that verapamil hypersensitivity and modulation of the MDR phenotype operate through different mecha-

nisms. This may possibly be due to the action of verapamil at more than one cellular target. Studies on other resistance modifiers with more specific cellular effects may clarify this point.

The difference in the responses of the two growth forms of VCR/T cells to verapamil could be considered either as a consequence of the failure of verapamil to penetrate the centre of the spheroids or as reflecting a physiological difference between cells grown by these two different techniques. We excluded the former possibility by showing that complete verapamil penetration occurs in a much shorter time than the period of verapamil treatment used to demonstrate lack of hypersensitivity, and we therefore propose that the observed difference in the hypersensitivity of VCR/T cells is due to cell physiological interactions occurring in spheroids but not in monolayers. It has previously been found that growth as multicellular spheroids induces altered mitoxantrone resistance in V79 cells [1] and that different levels of resistance to fluorouracil, tetraplatin and chlorambucil are expressed in different regions of V79 spheroids, independently of drug concentration [5], implying that a cellular microenvironment may play a major role in modulating the toxicity of these drugs. In the case of verapamil hypersensitivity, the cell surface would be the most obvious candidate as the site for the different cellular responses in spheroids and monolayers. Like most multidrug-resistant cell lines, VCR/T has elevated levels of a 180-kDa membrane protein [21], and in multidrug-resistant KB-cells, verapamil is known to interfere with photolabelling of this protein by vinblastine analogues [3]. It may be speculated that cells grown as three-dimensional spheroids would obviously have fewer exposed cell-surface components that may interact with verapamil than would cells grown as monolayers. Cell interactions that may influence the expression of verapamil hypersensitivity are of interest because they may have similarities to those that influence the more widespread phenomenon of verapamil reversal of multidrug resistance.

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