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PREVENTION OF VINBLASTINE-INDUCED CYTOTOXICITY BY RUTHENIUM RED*

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Abstract—Ruthenium red, an inorganic dye that reacts selectively with mucopolysaccharides of the plasma membranes, prevents vinblastine-induced cytotoxicity toward cultured KB cells. At 6.7 ng vinblastine/ml of the culture medium, the growth of KB cells was inhibited completely. When ruthenium red was present in the culture, however, the cytotoxicity induced by vinblastine was completely prevented at concentrations of vinblastine up to 67 ng/ml. Ruthenium red (6 μg/ml) decreased the intracellular amount of vinblastine, whereas in the absence of ruthenium red the level of cellular vinblastine was maintained for 6 hr. This rapid decrease of vinblastine can be explained by an inhibition of uptake of vinblastine through the plasma membrane, and not by an enhanced release of the drug from the cells. Ruthenium red specifically inhibited the Ca^{2+}-ATPase activity of the plasma membrane of KB cells. The possible involvement of this inhibition in the preventive effect of ruthenium red on vinblastine-induced cytotoxicity is discussed.

Microtubules and microfilaments are the primary components of the cytoskeletal structure in eukaryotic cells [1–3]. These components connect either directly or indirectly to macromolecules in the plasma membrane and are involved in the regulation of membrane-associated cellular events [2, 3]. Vinca alkaloids interact with the microtubular protein and arrest cell division in metaphase. The function of microtubules has been reported to be affected by Ca^{2+} [4–6]. It has been suggested that Ca^{2+} modifies the action of vinca alkaloids on cultured cells. And, in fact, ionophore A23187, which transports calcium and magnesium across a biologic membrane, inhibited the cytotoxicity of vinca alkaloids to leukemic lymphocytes to a moderate extent [7].

In this study, we examined the effects of ruthenium red, an inorganic dye, on the cytotoxicity of vinblastine to cultured KB cells. Ruthenium red reacts selectively with mucopolysaccharides of the plasma membranes [8] and inhibits Ca^{2+}-ATPase of the plasma membranes, leading to an inhibition of Ca^{2+} transport function across the membranes [9–11]. We found that ruthenium red prevented the vinblastine-induced cytotoxicity toward cultured KB cells. The dye specifically inhibited the Ca^{2+}-ATPase activity of the plasma membrane of KB cells, and also inhibited the uptake of vinblastine by cultured KB cells.

MATERIALS AND METHODS

Chemicals. Ruthenium red was purchased from the Sigma Chemical Co., St. Louis, MO, and was dissolved in 0.9% NaCl solution. [γ-^{32}P]ATP (tetratriethylammonium salt, 10–40 Ci/mmole) was purchased from the New England Nuclear Corp., Boston, MA. Vinblastine is a product of Eli Lilly and Co., Indianapolis, IN, and [^{3}H]vinblastine (10.4 Ci/mmole) was purchased from the Radiochemical Center, Amersham, Bucks., England.

Culture of KB cells and drug treatment. A clonal KB cell line [12] was grown in Eagle's minimal essential medium containing 10% calf serum (Grand Island Biological Co., Grand Island, NY). Cells were grown in plastic dishes (60 mm in diameter from Corning Glass Works, Corning, NY) containing 3 ml of the medium at 37° in a humidified atmosphere with 5% CO_2. Cells were re-cultured at a density of 1.5×10^5 cells/dish; 24 hr later the culture medium was replaced with fresh medium and the drug was added to the dishes. At 48 hr after the addition of the drug, the medium was removed, and the cell layer was washed with phosphate-buffered saline (PBS), trypsinized, and enumerated with a Coulter counter, as described previously [12]. Two dishes were used for each drug concentration; less than 5 per cent variation was noted between dishes.

Assay of Ca^{2+}-activated ATPase. Ca^{2+}-activated ATPase activity was calculated by subtracting the Mg^{2+}-dependent activity from the activity measured in the presence of Mg^{2+} and Ca^{2+} [10, 13, 14]. The KB cell suspension in 90 mM NaCl–50 mM KCl–30 mM Tris–acetate (pH 8.0) (25 μl, containing 5×10^4 cells) was added to the standard incubation mixture (175 μl) for [$Mg^{2+}+Ca^{2+}$]-activated ATPase containing 90 mM NaCl, 50 mM KCl, 30 mM Tris–acetate (pH 8.0), 0.1 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 0.2 mM ouabain. The reaction was started by adding 50 μl of 1.0 mM [γ-^{32}P]ATP (sp. act. 20–100 mCi/mmole), and the mixture was incubated at 37° for 10 min. The $^{32}P_i$ released from [$\gamma - ^{32}$P] ATP was measured as described previously [15]. Mg^{2+}-dependent ATPase activity was measured in the

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same manner as described above except that 2 mM ethyleneglycol-bis (aminoethyl)-tetraacetic acid (EGTA) was added in place of the $CaCl_2$. For the enzyme inhibition experiment, the cells were preincubated for 5 min in the standard incubation mixture containing ruthenium red, and the reaction was started by adding ATP.

Uptake of vinblastine by KB cells. KB cells were seeded in 100 mm plastic dishes containing 10 ml of the medium at a density of 5×10^5 cells/dish. At approximately 24 hr after seeding the cells, the radioactive vinblastine (1.3 ng/ml medium, sp. act. 10.4 Ci/mmole) was added, and the cells were cultivated as above. For the vinblastine-uptake inhibition experiment, ruthenium red and radioactive vinblastine were added successively to the culture. The density of the cells was approximately 8.5 to 9.0×10^5 cells per dish when the drugs were added. At various times after the addition of vinblastine, the cells were washed three times with PBS (each 10 ml) and trypsinized, and the radioactivity incorporated by the cells was counted as described previously [12]. Aliquots of cells were enumerated with a Coulter counter.

RESULTS

Inhibitory effect of ruthenium red on the proliferation of KB cells. Ruthenium red inhibited the growth of cultured KB cells (Fig. 1). At a lower concentration of the dye (2–6 μg/ml), the growth was inhibited by 70 per cent. With between 20 and 50 μg of the dye per ml of medium, the inhibitory effect was rather weak and only 40–50 per cent growth inhibition was observed. At higher concentrations (above 70 μg/ml), a stronger inhibition again occurred. We cannot explain this phenomenon at present. Between experiments, we observed approximately a 10 per cent variation in inhibition at each concentration of ruthenium red; however, the nonlinear inhibition by the dye always occurred as described above with cultured KB cells. Other cultured cell lines, such as mouse Lewis lung carcinoma and P388 leukemia, showed growth inhibition dependent upon drug concentration.

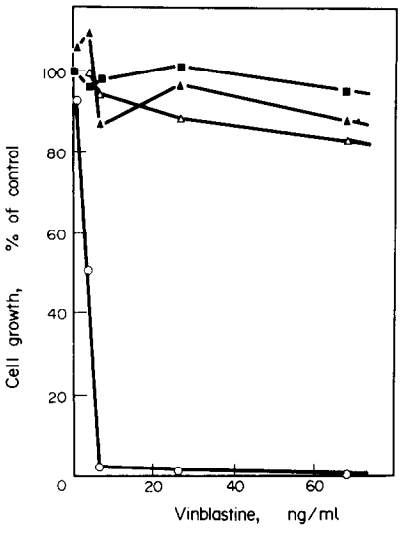

Fig. 2. Effect of ruthenium red on the growth of KB cells in the presence of vinblastine. Twenty-four hours after sub-culturing the KB cells, the cells were treated with ruthenium red at 70 (■), 30 (△), 6 (▲) and 0 (○) μg/ml of culture medium in the presence of vinblastine at the indicated concentrations of 67.0, 27.0, 6.7, 4.0 and 1.3 ng/ml of culture medium. One hundred per cent cell growth of KB cells in the absence of vinblastine at concentrations of ruthenium red of 70, 30, 6 and 0 μg/ml of culture medium corresponds to the cell numbers of 3.4, 4.3, 2.4 and 8.1×10^5 cells per dish.

Preventive effect of ruthenium red on vinblastine-induced cytotoxicity. Vinblastine efficiently inhibited the growth of cultured KB cells (Fig. 2). At 4.0 ng/ml growth was inhibited by approximately 50 per cent

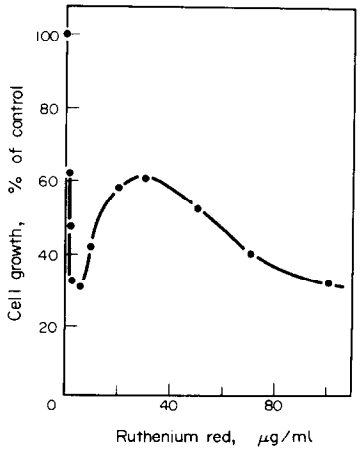

Fig. 1. Effect of ruthenium red on the growth of KB cells. KB cells were treated with ruthenium red at the indicated concentrations and cultivated in the presence of the dye as described in Materials and Methods. One hundred per cent cell growth corresponds to the cell number of 8.09×10^5 cells per dish.

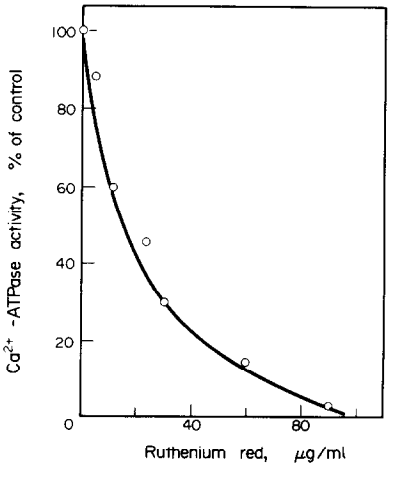

Fig. 3. Effect of ruthenium red on Ca^{2+}-ATPase activity. KB cells were preincubated for 5 min in the standard incubation mixture at the indicated concentrations of ruthenium red and the assay was carried out as described under Materials and Methods. One hundred per cent activity was 14.5 nmoles P_i liberated per 10^5 cells per 10 min.

and at 6.7 ng/ml growth was almost completely inhibited. When ruthenium red was added to the culture at 6, 30 and 70 μg/ml, however, the cytotoxicity induced by vinblastine was completely prevented up to a concentration of vinblastine of 67 ng/ml (Fig. 2). This preventive effect by the dye was consistently observed at the various concentrations (i.e. 6–70 μg/ml) of dye tested.

Inhibition of Ca^{2+}*-ATPase by ruthenium red.* Inhibition of Ca^{2+}-ATPase by ruthenium red is shown in Fig. 3. At 6, 12, 30, 60 and 90 μg of ruthenium red per ml of reaction mixture, the reaction was inhibited by 11.7, 39.5, 72.5, 85.3 and 94.8 per cent respectively. This was confirmed by time-dependent reactions. Ca^{2+}-ATPase activity was almost completely inhibited by 90 μg ruthenium red/ml of reaction mixture during the 20 min of incubation. Mg^{2+}-ATPase activity, however, was completely resistant to ruthenium red. This clearly indicates that ruthenium red is a specific inhibitor of Ca^{2+}-ATPase.

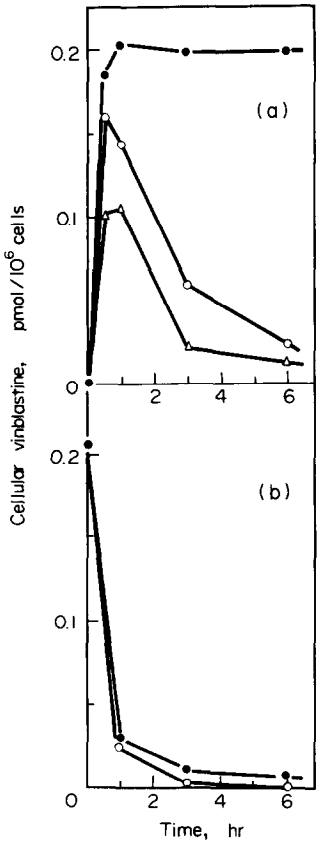

Fig. 4. Panel (a): Effect of ruthenium red on the cellular uptake of vinblastine. The cells were incubated with vinblastine (1.3 ng/ml) in the absence (●) or presence (○) of ruthenium red (6 μg/ml), and at fixed intervals cellular uptake of vinblastine was determined as described under Materials and Methods. In another experiment (△), cells were preincubated with ruthenium red for 3 hr, vinblastine was added, and cellular uptake of vinblastine was determined as above. Panel (b): Effect of ruthenium red on the release of vinblastine from the cells. KB cells were cultured in the presence of labeled vinblastine (1.3 ng/ml) for 1 hr. The medium was removed and the cells were washed with cold phosphate-buffered saline (10 ml). The cells were cultivated in fresh medium with non-labeled vinblastine (1.3 ng/ml) in the absence (●) or presence (○) of ruthenium red (6 μg/ml). At intervals, the labeled vinblastine retained in the cells was determined.

Inhibition of cellular uptake of vinblastine by ruthenium red. Approximately 0.2 pmole of vinblastine had been incorporated into 10^6 cultured KB cells by 1 hr after the addition of vinblastine (1.3 ng/ml) to the medium (Fig. 4A). This net incorporation was maintained during the next 5 hr. When ruthenium red (6 μg/ml) was added to the culture just prior to the addition of vinblastine, however, the incorporation of vinblastine reached a maximum within 0.5 to 1 hr, and a rapid decrease of intracellular vinblastine occurred thereafter. At 6 hr, the intracellular amount of vinblastine was approximately 0.03 pmole/10^6 cells. This amount was 15 per cent of that retained at 6 hr in the absence of ruthenium red. A similar decrease of vinblastine was observed at 30 and 60 μg of the dye per ml of the culture medium. More than 90 per cent of the cells that were treated with 6–60 μg of ruthenium red for 6 hr excluded trypan blue, suggesting an intactness of the plasma membrane.

When the cells were preincubated with ruthenium red for 3 hr, the inhibition of cellular uptake of vinblastine was more prominent (Fig. 4A). The cellular level of vinblastine was approximately 40–60 per cent of that observed when ruthenium red was added just prior to the addition of vinblastine. Labeled vinblastine incorporated into the cells was rapidly cleared from the cells when the medium was replaced with fresh medium containing non-labeled vinblastine (Fig. 4B). At 1 hr after the replacement of the medium, approximately 85 per cent of the labeled intracellular vinblastine disappeared from the cells, and at 3 hr more than 95 per cent of the labeled vinblastine was lost from the cells. Ruthenium red had no effect on the release of vinblastine from the cells. These two experiments indicate that the decrease of cellular levels of vinblastine by ruthenium red is caused by an inhibition of cellular uptake of vinblastine, and not by an enhanced release of the drug from the cells.

DISCUSSION

Ruthenium red almost completely prevented the cytotoxicity of vinblastine. The mechanism of this phenomenon may be explained by a decrease in the amount of intracellular vinblastine caused by a blockade of cellular uptake of the drug. Ruthenium red modifies the plasma membrane and consequently seems to inhibit the transport of vinblastine across plasma membranes. The preventive effect of ruthenium red on vinblastine-induced cytotoxicity could be explained by this mechanism. However, since it has been reported that an increase in uptake of Ca^{2+} reduces the cytocidal action of vinblastine against cultured lymphatic leukemia cells [7], we can speculate upon another mechanism. The inhibition of Ca^{2+}-ATPase of the plasma membrane by ruthenium red could lead to an increase in intracellular Ca^{2+}, and this increase of Ca^{2+} might reduce the cytotoxicity of vinblastine to cultured KB cells, as has been

observed in the case of cultured lymphatic leukemia cells [7]. We need to experiment further to confirm or deny the second possibility.

Strong cytotoxicity of ruthenium red was observed at 6 μg/ml of culture medium (Fig. 1), whereas the inhibition of Ca^{2+}-ATPase by ruthenium red was dose-dependent (Fig. 3). The cytotoxicity of ruthenium red toward cultured KB cells is not directly connected with inhibition of ATPase by the dye. It is of interest that ruthenium red did not show a dose-dependent cytotoxicity toward cultured KB cells, whereas the dye showed a dose-dependent toxicity toward cultured mouse cells such as Lewis lung carcinoma and P388 leukemia cells. The various mechanisms of this phenomenon remain to be resolved.

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