Potentiation of Invasive Capacity of Rat Ascites Hepatoma Cells by Adriamycin

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ABSTRACT

The effect of Adriamycin on the invasive capacity of rat ascites hepatoma cells, W1, was studied. The invasive capacity of W1 cells was estimated in vitro by counting the number of penetrated single tumor cells and tumor cell colonies formed from the penetrated cells underneath a cultured mesothelial cell monolayer (H. Akedo et al., Cancer Res., 46: 2416–2422, 1986). A considerable increase of the invasive capacity was observed when the tumor cells had been treated with 1.0 to 20.0 μM Adriamycin. This augmentation of invasive capacity of tumor cells was partially inhibited by 60 μM N-acetylcysteine, a scavenger of free radicals. On the other hand, 60 μM N-acetylcysteine did not impair the cytotoxicity of Adriamycin for W1 cells measured by an in vitro tetrazolium-based colorimetric assay for cytotoxicity.

INTRODUCTION

The antitumor antibiotic, Adriamycin, is one of the most effective drugs in the treatment of a number of human tumors. However, the mechanism of the action of Adriamycin still remains controversial. It is now known that the exposure to Adriamycin initiates several biochemical events, all of which may not be relevant to its cytotoxic action. Some investigators reported that the antitumor drugs including Adriamycin had such abilities as induction of tumors in experimental animals (1), transformation of cultured cell lines (2), and promotion of skin tumors (3). Another report showed that treatment of tumor cells with an antioxidant drug resulted in an enhanced metastasis formation in mice (4). These indicate that Adriamycin may alter tumor cells to be more aggressive.

The aggressiveness of tumor cells appears most prominently as their invasiveness and metastatic property. We have established a model experimental system to estimate the invasive capacity of tumor cells, in which rat ascites hepatoma cells were overlaid on a cultured mesothelial cell monolayer (5–8). The in vitro invasive capacities of tumor cells estimated in this system correlated well with their in vivo invasive abilities (7, 8). By using this system, we have shown that oxygen radicals (6), activated macrophages (7), and transforming growth factor β (8) potentiated the in vitro invasive capacity of tumor cells. In these studies, it was suggested that free radical generators might be involved in augmenting effect on tumor aggressiveness. Adriamycin is well known to generate free radicals (9, 10). In the present study, we found that Adriamycin potentiated the invasiveness of rat ascites hepatoma cells.

MATERIALS AND METHODS

Drugs and Chemicals. Adriamycin solution (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan; 0.75–20.0 μM) was prepared by dissolving the drug in 0.9% NaCl solution. N-Acetylcysteine (Sigma Chemical Company, St. Louis, MO) was dissolved in phosphate-buffered saline (NaCl, 8 g/liter; KCl, 0.2 g/liter; NaH2PO4·2H2O, 2.9 g/liter; and KH2PO4, 0.2 g/liter) immediately before use. SOD1 and CAT were purchased from Calzyme Laboratories, Inc., San Luis Obispo, CA, and Boehringer Mannheim GmbH, West Germany, respectively.

Cells and Cell Culture. Rat M-cells were isolated from Donryu rats (Japan SLC, Inc., Hamamatsu, Japan) mesentery and cultured in MEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% FCS (Cell Culture Laboratories, Cleveland, OH), as reported previously (5). When M-cells grew to confluence (M-cell monolayer), the layer was used for the assays of invasive capacity. Rat ascites hepatoma (AH 130) cells (a gift from Biomedical Research Center, Osaka University, Medical School) that had been maintained in the peritoneal cavity of male Donryu rats were cultured in MEM supplemented with 10% FCS in an atmosphere of 95% air and 5% CO2 at 37°C. The poorly invasive clone, W1, was isolated from the cultured parental AH 130 cells by limiting dilution and maintained as suspension culture (8).

Treatment of W1 Cells with Adriamycin. W1 cells (1.0 × 106/ml) were cultured in MEM supplemented with 10% FCS for various lengths of time in the presence of Adriamycin. After the treatment, the cells were washed once with the fresh culture medium and seeded on M-cell monolayers.

pretreatment of W1 Cells with Scavengers. W1 cells were pretreated for 30 min with either N-acetylcysteine (60 and 120 μM) or SOD (75.0 and 112.5 units/ml) and CAT (62.5 and 93.8 units/ml); then they were exposed to Adriamycin in the presence of the scavengers. After the Adriamycin treatment, cells were washed and resuspended in the culture medium containing the scavengers.

Assays. The assay procedure of the in vitro invasive capacity of W1 cells was essentially the same as described in our previous reports (5, 6). W1 cells (2.0 × 105 cells/dish) were seeded on a M-cell monolayer and cultured. The tumor cells penetrated a M-cell layer as independent single cells, each of which then proliferated under the M-cell layer to form a colony (5). Forty h after the tumor cell seeding, the culture supernatant was removed and the residual cells on the dish were fixed in situ in 10% formol. The number of penetrated single tumor cells and colonies formed under the M-cell monolayer was counted in 45 different visual fields (0.13 mm2 each) under a phase contrast microscope and calculated as penetrated single cells and colonies/cm2. Since W1 cells appeared in the form of an independent single-cell suspension in culture, each penetrated colony was developed from a single penetrated tumor cell; therefore, the number of penetrated single cells and colonies/cm2 was defined as the in vitro invasive capacity.

The cytotoxicity of Adriamycin was determined by MTT assay (11, 12) with a slight modification. W1 cells (5.0 × 104/ml) treated for 1 h with Adriamycin were seeded in a well of a 24-well culture plate (Linbro 76063079). After 96-h culture, 150 μl of each of cultured cell suspension were transferred into triplicate wells of a 96-well microtiter plate (Corning 25850), and then 25 μl of MTT (Sigma) solution were added to each well. Three h later, the concentration of colored formazan products was measured spectrophotometrically at 550 nm.

Statistics. Data were analyzed either by a continuity-adjusted χ2 test for comparison of two counts (Tables 1, 2, and 4) or by analysis of variance (Table 3).

RESULTS

In Vitro Invasive Capacity of W1 Cells Treated with Adriamycin. W1 cells, when treated with Adriamycin, penetrated a...
M-cell monolayer more extensively than the untreated W1 cells, indicating the potentiation of the invasive capacity of tumor cells by Adriamycin (Table 1). This potentiation depended on the concentration of Adriamycin: a significant increase was observed at 1.0 µM Adriamycin and the maximum increase at 6.0–10.0 µM Adriamycin. As shown in Table 2, this potentiation also depended on the time period of treatment; the maximum effect was obtained when W1 cells were treated for 2–3 h with 3.0 µM Adriamycin.

From the above results, we assumed that treatment with 6 µM Adriamycin for 1 h could result in about 50% induction of invasiveness. Thus we tested the tumor cell viability after treatment for 1 h with 0.75–12.0 µM Adriamycin. More than 0.75 µM Adriamycin showed an appreciable cytotoxic activity depending on the concentration of the drug (Table 3). Therefore, the potentiation of invasiveness and the viability of tumor cells after treatment with Adriamycin appear to correlate inversely.

<table>
<thead>
<tr>
<th>Concentration of Adriamycin (µM)</th>
<th>No. of penetrated single cells and colonies/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>18 ± 4*</td>
</tr>
<tr>
<td>1.0</td>
<td>57 ± 3*</td>
</tr>
<tr>
<td>3.0</td>
<td>134 ± 11</td>
</tr>
<tr>
<td>6.0</td>
<td>211 ± 36</td>
</tr>
<tr>
<td>10.0</td>
<td>193 ± 21</td>
</tr>
<tr>
<td>20.0</td>
<td>169 ± 32</td>
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</tbody>
</table>

* Mean ± SD of at least 3 determinations.

<table>
<thead>
<tr>
<th>Period of incubation (h)</th>
<th>No. of penetrated single cells and colonies/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>33 ± 16*</td>
</tr>
<tr>
<td>0.5</td>
<td>58 ± 3*</td>
</tr>
<tr>
<td>1.0</td>
<td>92 ± 10</td>
</tr>
<tr>
<td>2.0</td>
<td>237 ± 29</td>
</tr>
<tr>
<td>3.0</td>
<td>206 ± 18</td>
</tr>
</tbody>
</table>

* Mean ± SD of at least 3 determinations.

The present study showed the change of aggressive behavior of tumor cells, the potentiation of in vitro invasive capacity, by exposure to Adriamycin. This effect was distinctly observed even when the tumor cells were treated with 1.0 µM Adriamycin for 3 h. The measured invasive capacities showed some extent of interassay variations. These appear to have resulted from the different preparations of M-cells, because the primary cultured M-cell monolayers were prepared each time for each group of experiments.

Van Putten et al. (13) showed that several cytotoxic agents including Adriamycin increased the formation of metastatic tumor nodules of osteosarcoma C22LR in the lung of mice. Geldof and Rao (14) recently reported that Adriamycin injected

<table>
<thead>
<tr>
<th>Concentration of Adriamycin (µM)</th>
<th>Adriamycin-treated</th>
<th>Adriamycin + NAC-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.302 ± 0.010* (100.0)*</td>
<td>0.364 ± 0.014&quot; (100.0)</td>
</tr>
<tr>
<td>0.75</td>
<td>0.258 ± 0.019&quot; (85.44)</td>
<td>0.286 ± 0.011* (78.6)</td>
</tr>
<tr>
<td>1.5</td>
<td>0.240 ± 0.006* (79.5)</td>
<td>0.231 ± 0.007&quot; (63.5)</td>
</tr>
<tr>
<td>3.0</td>
<td>0.189 ± 0.029&quot; (62.6)</td>
<td>0.203 ± 0.016* (55.8)</td>
</tr>
<tr>
<td>6.0</td>
<td>0.152 ± 0.001&quot; (50.3)</td>
<td>0.150 ± 0.023* (41.2)</td>
</tr>
<tr>
<td>12.0</td>
<td>0.135 ± 0.028&quot; (44.7)</td>
<td>0.156 ± 0.021* (42.9)</td>
</tr>
</tbody>
</table>

* 60 µM N-acetylcysteine.

Since the cytotoxicity of 0.75 to 6.0 µM Adriamycin appears to be at most 50% (Table 3), more than one-half of the penetrated tumor cells were assumed to survive at these treatment conditions. To ascertain this assumption, the tumor cells were treated with 6 µM Adriamycin for 1 h and seeded on a M-cell monolayer, and the resultant penetrated tumor cells were further cultured. About 40% of the penetrated cells survived at least for 11 days, and 56% of them were proliferated. The number of proliferated colonies was 4–5 times as much as that of the untreated control (data not shown).

Effect of Scavengers. The pretreatment of W1 cells with 60 µM N-acetylcysteine, a radical scavenger, revealed about 60% inhibition of the potentiation of invasive capacity of the cells by Adriamycin (Table 4). The same concentration of N-acetylcysteine showed almost no effect on the invasiveness of untreated W1 cells. Pretreatment of W1 cells with 75.0 units/ml SOD and 62.5 units/ml CAT also produced a small but statistically significant inhibition of the potentiation of the invasive capacity of W1 cells by Adriamycin. The inhibition by 60 µM N-acetylcysteine was ranged from 30 to 60% in the repetitive experiments. The trend of inhibition was, however, consistent. Pretreatment with 120 µM NAC or 112.5 units/ml SOD and 93.8 units/ml CAT exhibited a similar level of suppression as above, respectively (data not shown). Neither 2 nor 10 µM mannitol, another antioxidant, showed the inhibitory effect (data not shown).

NAC treatment per se increased the absorbance value in MTT assay in the absence of Adriamycin. The reason for this is not clear. However, the cytotoxicity of Adriamycin measured by MTT assay was not impaired by treatment with N-acetylcysteine (Table 3).

DISCUSSION

The present study showed the change of aggressive behavior of tumor cells, the potentiation of in vitro invasive capacity, by exposure to Adriamycin. This effect was distinctly observed even when the tumor cells were treated with 1.0 µM Adriamycin for 3 h. The measured invasive capacities showed some extent of interassay variations. These appear to have resulted from the different preparations of M-cells, because the primary cultured M-cell monolayers were prepared each time for each group of experiments.

Van Putten et al. (13) showed that several cytotoxic agents including Adriamycin increased the formation of metastatic tumor nodules of osteosarcoma C22LR in the lung of mice. Geldof and Rao (14) recently reported that Adriamycin injected
ADRIAMYCIN-INDUCED POTENTIATION OF INVASION

i.v. caused a significant increase in the metastasis of a prostate tumor to the lung in mice. McMillan and Hart (15) showed that tumor cells treated with antitumor drugs in vitro also acquired a highly metastatic potential. Metastasis develops by a multiprocess including the invasion and local growth; their findings did not specify which one of the steps was affected by Adriamycin. In the present study, we demonstrated that Adriamycin potentiated the invasive capacity of W1 cells. Similarly, the invasive capacity of MM1, another cell line with the high invasive potential derived from AH 130 cells, was also enhanced by the treatment with Adriamycin, although to a lesser extent. The treatment of W1 cells with 6.0 μM Adriamycin for 1 h resulted in at least a 4-fold increase in the penetrated proliferative tumor cells. This suggests that the treatment with Adriamycin that does not eliminate all tumor cells may increase the incidence of invasion and metastasis, although the number of total tumor cells would be decreased by the drug.

Solid tumor in general evolves from one cell to a clinical entity exhibiting pronounced heterogeneity (16). The phenotype of tumors usually changes over a period of time to be more aggressive. This acquisition of more aggressive traits of neoplastic cells, tumor progression, is of particular importance because highly infiltrative and metastatic clones appear to become predominant in this process. Extrinsinc factors are likely to participate in this process, whereas the genetic instability of neoplastic cells is one of the most essential contributions. Activated macrophages and transforming growth factor β were shown to give certain tumor cells a more aggressive phenotype in our previous study (7, 8). Kerbel and Davies (17) have presented the idea that the use of antineoplastic drugs can cause tumor progression. There are also several clinical reports that suggest the increased incidence of distant metastases with a distinct reduction of primary tumors after the treatment with antineoplastic drug (18, 19). Our present results suggest the possible involvement of Adriamycin in tumor progression.

Green et al. (20) showed a biexponential disappearance kinetics of Adriamycin given to patients: the concentration of Adriamycin in plasma exhibited an initial rapid decline from 5.0 to 0.1 μM within 1 h following 15-min infusion. Such plasma concentrations of the drug are in the near range of those used in the present study. This suggests a possible enhancement of malignancy in the clinical usage of Adriamycin, if not all. Moreover, even milder exposure may produce a similar phenomenon for more sensitive cells, because W1 cells seemed less sensitive to Adriamycin.

The augmentation of invasive capacity by Adriamycin was considered to be partly mediated by free radicals generated by Adriamycin, because N-acetylcysteine partially suppressed it. Much less effectiveness of SOD and CAT on the prevention of Adriamycin-induced potentiation of invasion may imply that the free radical generation in the cells is essentially involved in its potentiating effect. This is consistent with the well-established fact that quinones may undergo reduction via intracellular reductases to either semiquinones or quinols which may subsequently react with molecular oxygen to yield O₂ and H₂O₂ (21).

The relationship between free radical formation and cytotoxicity of Adriamycin and the effect of radical scavengers on the cytotoxicity of Adriamycin are still controversial; several investigators have reported that free radical production plays a critical role in the cytotoxicity of Adriamycin, but others failed to obtain any evidence for it. SOD and CAT have been reported to inhibit the cytotoxicity of Adriamycin (22), whereas N-acetylcysteine or cysteamine was reported to be ineffective in certain experimental systems (23). In the present study, N-acetylcysteine did not impair the cytotoxicity of Adriamycin for W1 cells, while it suppressed the Adriamycin-induced potentiation of invasiveness. These facts suggest that the mechanism by which Adriamycin exerts cytotoxicity is different from that of its action on invasiveness. This may give us a new rationale for Adriamycin therapy in combination with radical scavengers to avoid its unfavorable effect, without impairing its cytotoxic activity. The reason for differential actions of NAC is not clear. The site responsible for induction of the potentiation of invasiveness may differ from that for Adriamycin cytotoxicity; the effective concentration of free radicals may be different in each action that can be discriminated by the pretreatment with NAC. The alternative possibility is that the distribution of the added NAC in the cell may not be uniform and NAC can better scavenge free radicals contributing to the increase of invasiveness. Okamoto et al. (24) showed the differential effects of the antioxidants, α-tocopherol and riboflavin, on the Adriamycin-induced lipid peroxidation and the suppression of DNA synthesis by Adriamycin.

Our present results are, however, based on the in vitro experiments using animal tumor cells. The interaction between tumor cells and chemotherapeutic agents in patients is probably more complex, and human tumor cells appear to contain different amounts of scavengers from those of animal cells (25). Much work will be necessary for the complete understanding of the action of Adriamycin in vivo. Nevertheless, it seems important to reevaluate the clinical use of free radical generators, such as certain anticancer drugs and radiation, in combination with radical scavengers for future successful chemoradiotherapy.

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REFERENCES


* Unpublished results.
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