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Report

Overexpression of aldose reductase in liver cancers may contribute to drug resistance

Karen WY Lee,[1] Ben CB Ko,[1] Zhirong Jiang,[1] Deliang Cao[1] and Stephen SM Chung[1]
[1]Institute of Molecular Biology, The University of Hong Kong, Pokfulam, Hong Kong, China.

We previously found that about 29% of human liver cancers overexpressed aldose reductase (AR) and about 54% of them overexpressed an AR-like gene called ARL-1 that has similar enzymatic activities to AR. Since these aldo–keto reductases can reduce a broad spectrum of substrates including cytotoxic aldehydes, we were interested to find out if these enzymes can contribute to the resistance of liver cancer chemotherapy by inactivating some of the anticancer drugs. HepG2 cells, a stable line of liver cells, were induced to overexpress AR by hypertonicity. Cells that were cultured in hypertonic medium became more resistant to daunorubicin, suggesting that overexpression of AR made the cells more resistant to this drug. This is confirmed by the fact that addition of AR inhibitor sensitizes the cells to this drug again. This information may be important for designing new drugs to treat this deadly disease. [© 2001 Lippincott Williams & Wilkins.]

Key words: Aldose reductase, daunorubicin, drug resistance, hepatocellular carcinoma, liver cancer.

Introduction

It is well known that liver cancer [hepatocellular carcinoma (HCC)] is resistant to a number of anticancer drugs; however, the reason is not entirely clear. The multidrug resistant (MDR) gene encoding P-glycoprotein (P-gp) is most likely involved. P-gp is a membrane protein that is able to transport a number of anticancer drugs of unrelated structures out of the cells.[1] Overexpression of this gene leads to an efflux of the anticancer drug, reducing its efficacy. However, this MDR gene is most likely not the only factor contributing to drug resistance. The profile of drug resistance in liver cancers is too complex to be explained by the overexpression of MDR gene alone.[2]

Aldose reductase (AR) was first identified as the enzyme that reduces glucose to sorbitol. It has received a lot of attention because of its involvement in the etiology of diabetic complications.[3] Its physiological function, however, is not clear. It is thought to be responsible for the production of fructose in the testis, because sorbitol can be converted by sorbitol dehydrogenase to fructose.[4] In the kidney, AR is thought to be involved in osmoregulation, because sorbitol can serve as an osmolyte and high salt induces the expression of this gene with a concomitant increase in sorbitol.[5] Besides testis and kidney, AR is expressed in most other tissues where its function is unknown. Glucose is in fact not its preferred substrate (K_m=100 mM, k_{cat}/K_m=2.8×10^2 M^{-1} min^{-1}).[6] It is more effective in reducing a large variety of aromatic and aliphatic aldehydes.[7] AR has been shown to reduce methylglyoxal (K_m=7.8 μM, k_{cat}/K_m=1.8×10^7 M^{-1} min^{-1}),[8] a toxic product of glucose metabolism, and 4-hydroxynonenal (K_m=22 μM, k_{cat}/K_m=4.6×10^6 M^{-1} min^{-1}),[9] a toxic product of lipid peroxidation, suggesting that its main function in the cell is to reduce these and other cytotoxic aldehydes.

AR was found to be induced in rat hepatomas and it was suggested that it might serve to inactivate toxic metabolites generated by the fast-growing cancer cells.[10] We found that about 29% of human liver cancers overexpress AR and about 54% of them overexpress an AR-like protein whose amino acid sequence is 70% identical to that of AR.[11] The cDNA of this protein called ARL-1 was expressed in *Escherichia coli*, and the recombinant ARL-1 was found to have similar enzymatic activities to AR in that it can reduce a variety of aromatic and aliphatic aldehydes. Since the related enzymes aldehyde reductase and ketone reductase had been shown to detoxify daunorubicin,[12]

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Correspondence to SSM Chung, Institute of Molecular Biology, The University of Hong Kong, Pokfulam, Hong Kong, China.
Tel: (+852) 22990782; Fax: (+852) 28171006;
E-mail: smchung@hkucc.hku.hk

we wanted to find out if overexpression of AR and ARL-1 would make the cells more resistant to this anticancer drug.

Materials and methods

Chemicals and media

Daunorubicin was obtained from Calbiochem (La Jolla, CA). Betaine was purchased from Sigma (St Louis, MO). AR inhibitor (AL1576) was a gift from Alcon Laboratories (Fort Worth, TX). MEM medium, fetal bovine serum, penicillin, streptomycin, non-essential amino acids, glucose and sodium pyruvate were obtained from Life Technologies (Gaithersburg, MD). Cytotoxicity detection kit (LDH) was purchased from Roche Molecular Biochemicals (Mannheim, Germany).

Cell cultures

HepG2 cells were obtained from ATCC (Rockville, MD). Isotonic cell culture medium was made up of MEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM MEM non-essential amino acids and 1 mM sodium pyruvate. Daunorubicin was dissolved in double-distilled water to make 10 mg/ml stock solution. AR inhibitor AL1576 was dissolved in 20 mM NaOH to a final concentration of 20 mM. Cells were first cultured in isotonic medium until 70% confluent. Then, the medium was made hypertonic by adding NaCl to 100 mM. The osmolarity of the medium, determined by an osmometer, was approximately 500 mosmol/kg. The cells were incubated in the hypertonic medium for 72 h, harvested and seeded in 24-well plates at 8×10^4 cells/well. After 24 h the cells were switched to hypertonic medium without serum and daunorubicin was added as indicated. Isotonic control cells were cultured in parallel but without being exposed to hypertonic medium.

LDH assays

Two hours after the addition of daunorubicin, the amount of LDH released to the medium was determined by the cytotoxicity detection kit. The optical density of the colored product was measured at 492, as suggested by the manufacturer, using a SpectraMax 340 Microplate reader (Molecular Devices, Sunnyvale, CA). LDH released into the medium is presented as a percentage of total LDH which was estimated by using the same assay kit to determine the LDH activity in cell extracts prepared by lysing the cells with 1% Triton X-100.

Northern blot hybridization

Samples of 20 μg of total RNA were loaded on 1.2% agarose gel containing 3% formaldehyde. After electrophoresis the RNA was transferred onto Hybond N+ membranes (Amersham, Little Chalfont, UK) by capillary blotting in $20 \times$ SSC (3 M NaCl and 0.3 M sodium citrate). The membranes were first incubated with prehybridization buffer at 65°C for 1 h, and then changed to hybridization buffer with α-^{32}P-labeled cDNA probes and incubated at 65°C for 12 h. The filters were then briefly washed twice with a solution of $0.1 \times$ SSC and 0.5% SDS at room temperature, and twice in the same solution for 30 min each at 65°C. The radioactivity on the filters was visualized by the PhosphoImager system (Molecular Dynamics, Sunnyvale, CA); for better quality images, the filters were also exposed to X-ray film (X-OMAT AR; Eastman Kodak, Rochester, NY) at −70°C with an intensifying screen.

Results

To simulate overexpression of AR in HCC, we induced the expression of AR in HepG2 cells, a human liver cell line, by hypertonicity. Similar to many other cells studied, HepG2 cells have been shown to induce the expression of AR by about 15-fold in hyperosmotic medium.[17] As shown in Figure 1, there was a large increase in the 1.35 kb AR mRNA after 24 h incubation in the hypertonic medium. The high level of this mRNA persisted throughout the remaining course of the 96-h hypertonic treatment. There was a larger 2.37 kb mRNA that was transiently induced between 12 and 24 h. This is the incompletely processed AR mRNA described earlier.[17] We used this cell culture system to see if overexpression of AR contributes to drug resistance.

The anticancer drug daunorubicin was shown to be a substrate for aldehyde reductase,[12] an enzyme whose

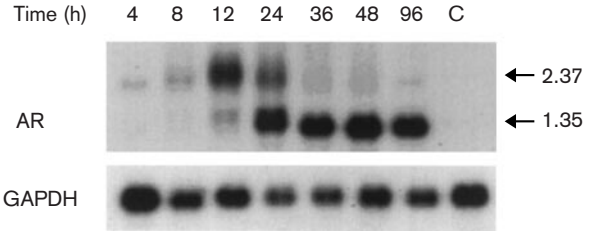

Figure 1. Induction of AR gene expression in HepG2 cells at various times in hypertonic medium. The 2.37 kb band represents an incompletely processed AR RNA. The 1.35 kb band is the functional AR mRNA. GAPDH is used to indicate the relative amounts of RNA loaded onto each lane.

amino acid sequence is about 50% identical to that of AR and whose spectrum of substrates also overlaps with that of AR. We therefore wanted to see if overexpression of AR in HepG2 cells would make them more resistant to this drug. Cells cultured in hypertonic medium, as well as control cells maintained in isotonic medium, were treated with 10 or 100 μg/ml of daunorubicin. The amounts of LDH released into the medium are taken as a measure of the cytotoxicity of this drug. As shown in Figure 2, 10 μg/ml of this drug has no significant effect on the cells. On the other hand, after 2 h incubation in the presence of 100 μg/ml of daunorubicin, approximately 75% of the LDH activity was released from the cells cultured in isotonic medium, indicating that this drug is killing about 75% of the cells during that period. Cells grown in hypertonic medium are more resistant to this drug. Only about 35% of the cells were dead under the same treatment.

To see if the increased resistance to daunorubicin is due to increased expression of AR, the HepG2 cells cultured in hypertonic medium were treated with an AR inhibitor AL1576. As shown in Figure 2, addition of the AR inhibitor (ARI) sensitizes these cells to daunorubicin, indicating that the increased resistance to this drug is indeed due to the overexpression of AR induced by hypertonic medium. ARI also made the cells cultured in isotonic medium more sensitive to daunorubicin. This is not because the ARI is toxic to the cells. In the absence of daunorubicin, or in the presence of 10 μg/ml of this drug, ARI has no toxic effect on cells cultured in isotonic or hypertonic media. Thus, ARI only enhanced the toxic effect of daunorubicin, presumably by preventing AR from detoxifying it. ARI also sensitized the HepG2 cells cultured in isotonic medium towards daunorubicin. This is most likely because it suppressed the AR activity normally present at low levels in these cells.

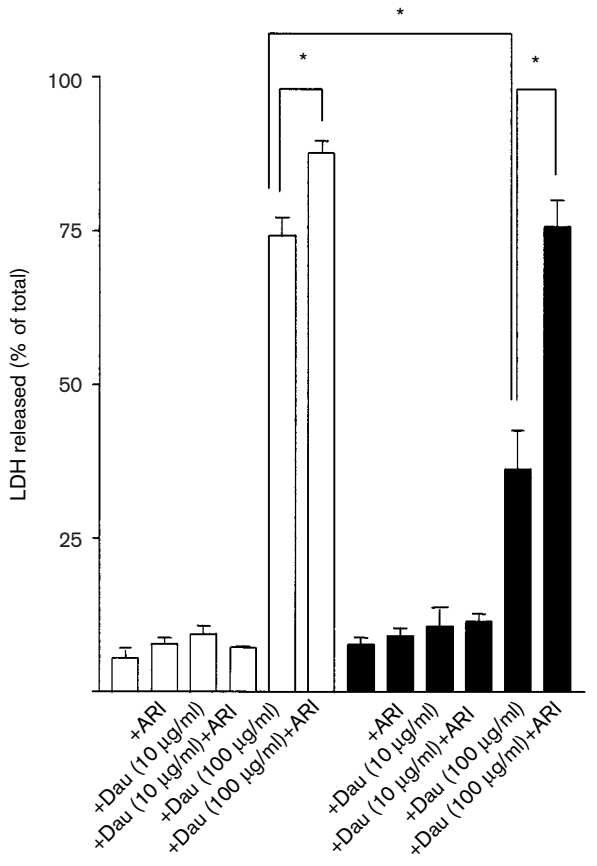

Figure 2. Cytotoxicity of daunorubicin (Dau). HepG2 cells were cultured in either isotonic (open columns) or hypertonic (filled columns) medium. Data are expressed as mean ± SD, with n=3. Statistical analysis was performed by one-way ANOVA (*$p < 0.001$).

Discussion

It is well known that HCC are resistant to a variety of anticancer drugs. However, different HCCs have different profiles of drug resistance. In a study using succinate dehydrogenase activity of tumor tissues as an assay to determine their response to various drugs, it was found that 47.2% of the HCC were sensitive to adriamycin (doxorubicin), 53.5% to mitomycin C, 10.3% to 5-fluorouracil, 51.5% to cisplatin, 51.5% to aclacinomycin A and 52.9% to carboquone. Among these HCC, 8% were sensitive to all drugs, 36.5% were resistant to all drugs and 55.5% were sensitive to only some of the drugs.[2] It is not clear if the results of these *in vitro* studies reflect drug response in patients. Nevertheless, it is clear that there are great variations in the drug response phenotype among the HCC, indicating that there are several mechanisms leading to their resistance to different drugs. One of the mechanisms is most likely the overexpression of MDR1. This gene, which codes for a glycoprotein responsible for the efflux of various hydrophobic molecules, including anticancer drugs,[16] was found to be expressed in 57% of the HCC.[1] However, overexpression of MDR1 alone cannot account for the diversity of drug-resistant phenotypes found in HCC.

We reported earlier that 29% of HCC overexpressed AR and 54% overexpressed a novel ARL-1, a novel protein whose amino acid sequence is 71% identical to that of AR.[11] Since AR can reduce a variety of aromatic and aliphatic aldehydes, and it is thought to be involved in detoxifying various cytotoxic aldehydes,[8–10] we were interested to find out if overexpression of these two genes in HCC may contribute to their resistance to various anticancer drugs. In this report

we showed that HepG2 cells that have higher AR activity are more resistant to daunorubicin, suggesting that AR could contribute to drug resistance in HCC. Although overexpression of AR was achieved by culturing the cells in hypertonic medium, the resistance to this drug is unlikely to be due to hypertonicity itself because AR inhibitors can reverse the resistance, indicating that the increase resistance to daunorubicin is due to increased AR activity.

In human HCC, ARL-1 is overexpressed more often than AR and therefore it will be important to find out if ARL-1 also contributes to drug resistance. However, hypertonicity does not induce the expression of ARL-1 in HepG2 cells (data not shown) and therefore we cannot use this system to test this enzyme. Since AR and ARL-1 have very similar spectrum of substrates, one would expect that overexpression of ARL-1 may also contribute to detoxification of daunorubicin. This drug is no longer used for the treatment of liver cancers. Here, we demonstrated that ARI significantly enhances its cytotoxic effect. Perhaps the use of this drug can be revived with the combined treatment with ARI. Both AR and ARL-1 can act on a broad spectrum of substrates. The overexpression of these two enzymes in liver cancers may lead to the inactivation of other anticancer drugs besides daunorubicin. Therefore, future development of drugs to treat this deadly disease should take this into consideration.

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