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Modification of Gene Products Involved in Resistance to Apoptosis in Metastatic Colon Cancer Cells: Roles of Fas, Apaf-1, NFκB, IAPs, Smac/DIABLO, and AIF

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Background. **Colon cancer becomes resistant to apoptosis as it acquires metastatic potential. SW480 and SW620 colon cancer cells were established from the same patient at different stages of tumor progression. The stage III colorectal cancer cell line (SW620) is more resistant to apoptosis. In the present report, we investigated the apoptotic gene products that might account for colon cancer evasion of immune attack and chemoradioresistance-induced apoptosis.**

Methods. **SW480 and SW620 cells were used for this experiment. Type 1 apoptosis was induced by CH-11. Type 2 apoptosis was induced by cisplatin and ionizing radiation. Apoptosis was determined by caspase-3 activity and terminal deoxynucleotidyl transferase mediated dUTP nick end labeling. Gene products Fas, TRAIL, c-FLIP, Bid, BAX, Bcl-2, Bcl-xL, Apaf-1, nuclear factor-kappa B, Smac/DIABLO, apoptosis inducing factor, and the inhibitors of apoptosis were investigated by immunocytochemistry and Western blot analyses.**

Results. **SW620 cell lines were more resistant to both Type 1 and Type 2 apoptosis induced by CH-11, cisplatin, and ionizing radiation, respectively. Examination of the extrinsic pathway demonstrated Fas receptor to be down-regulated in SW620. Apaf-1 was decreased in SW620 cells; while other members of the mitochondrial pathway including Bax, Bid, Bcl-xL, and Bcl-2 demonstrated minimal alterations of protein levels in both cell lines. Survivin and XIAP protein levels were increased in SW620 cells, which correlated with nuclear expression of nuclear factor-kappa B in SW620 cells but not SW480. Mitochondrial-released factors including Smac/DIABLO and apoptosis inducing factor were increased in SW480 cells.**

Conclusions. **SW620 cells have acquired genetic defects both in the intrinsic and extrinsic pathways of apoptosis, which may explain in part the ability of colon cancer cells to escape the immune system and to become chemoradioresistant. These genes may be potential targets for chemoradiosensitization in advanced colorectal cancer.**

Key Words: **SW480; SW620; caspases; apoptosis inducing factor; inhibitors of apoptosis; colon cancer; rectal cancer; radiation resistance; chemoresistance.**

INTRODUCTION

Since the vast majority of cytotoxic modalities exert their antitumor effects by induction of apoptosis, programmed cell death has rapidly emerged as a potential target for cancer treatment at various stages of tumor progression [1]. Immunoregulation and chemoradiosensitization are potential targets where insight in apoptotic mechanisms may lead to improvement of chemoradiotherapeutic modalities. In colon cancer, apoptosis decreases as the colonocyte progresses in the adenoma to carcinoma sequence of colon carcinogenesis and metastasis [2].

The extrinsic pathway of apoptosis is important in the elimination of unwanted cells by the immune system. Analysis of how defects in the extrinsic pathway of apoptosis permit colon cancer cells to escape the immune system may provide treatment options whereby

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the body's immune system could again recognize and eliminate unwanted cells [3, 4].

The intrinsic pathway of apoptosis plays a pivotal role in the elimination of damaged cells that have undergone intracellular stress [2]. Defects in this pathway of apoptosis, therefore, may lead to chemoradioresistance. The study of apoptosis in colon cancer has been dependent on *ex vivo* systems that demonstrate apoptosis at a single point in time. *In vitro* systems have investigated only a few apoptotic mediators at a time in different colon cancer cell lines [2, 5–7]. It is difficult to determine how changes of one mediator of apoptosis will affect the fate of other proapoptotic or antiapoptotic molecules in the entire process of programmed cell death when more than one cell line is examined. Examination of a single system where defects in the various pathways of apoptosis are established would permit the study of how chemotherapeutic interventions alter the entire cascade of apoptosis.

An *in vitro* model of colon carcinogenesis has been validated to represent the progression of colon carcinogenesis from a primary tumor to metastatic disease [8]. The SW480 cell line was derived from a primary, Dukes' stage B (colon adenocarcinoma) tumor from a 50-y-old Caucasian male. The SW620 cell line was cultured from a lymph node metastasis in the same patient at a later time [9, 10]. Because these cells are derived from the same patient, the gene product modifications that have occurred might be the result of changes the cells acquire as they progress into a metastatic phenotype. The aims of the present study are: (1) to demonstrate the relative sensitivity of Fas-, cisplatin-, and ionizing radiation-induced apoptosis between SW480 and SW620 cells; and (2) to investigate the specific gene modifications that might account for the observed differences in the rate of apoptosis between these two cell lines.

MATERIALS AND METHODS

Cell Culture and Reagents

Scott and White (SW)480 and (SW)620 human colon cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA): SW480 (colon adenocarcinoma CCL-228 passage number: 96) and SW620 (colon adenocarcinoma, lymph node metastasis CCL-227 passage number: 83). These cell lines were maintained at 37°C and 5% CO_2, in DMEM (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 1% (vol/vol) L-glutamine. For every experimental condition, the cells were cultured in 1% fetal bovine serum for 24 h prior to experimental treatment. Cells were grown and handled as previously described [11–14].

Induction of Apoptosis

For Type 1 apoptosis we elected to induce our cells with CH-11, which is a monoclonal antibody against Fas receptor. Apoptosis was then determined following treatment with CH-11. Type 2 apoptosis was induced by treatment of cells with cisplatin and ionizing radiation (IR).

Induction of Apoptosis with CH-11

SW480 and SW620 cells were plated and synchronized. Cells were either treated or untreated for 24 h with 40 and 100 ng/mL of CH-11 monoclonal antibody (Lake Placid, NY) as previously described [14]. These cells were then submitted to flow cytometry to determine the rate of apoptosis by caspase-3 activity.

Induction of Apoptosis with Cisplatin (CDDP)

Cis-diamminedichloroplatinum (II) (cisplatin or CDDP) is a chemotherapeutic agent that induces apoptosis by DNA-platination, or formation of cisplatin-DNA adducts. This DNA damage induces a mitochondrial response and the release of proapoptotic factors [15]. Because cisplatin is a potent inductor of apoptosis, we elected to use this therapeutic agent in this experimental protocol.

Cisplatin treatment was performed as in previous experiments [14] with minor modifications. SW480 and SW620 cells were plated and synchronized to achieve concordance. These cells were incubated for 48 h in the presence or absence of 5 and 10 μg/mL of cisplatin (Sigma-Aldrich, St. Louis, MO). Apoptosis was measured by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL).

Induction of Apoptosis with IR

Cells were treated with ionizing radiation as previously described [16]. Three hundred live cells, counted with a CASY cell counter, were seeded onto 60 mm dishes, and then left in the incubator for 6 h to attach. After 6 h, cells were irradiated at room temperature with doses of 0, 2, 4, and 6 Gy at a dose rate of 3.47 Gy/min^{-1}, using a Mark 1 JL Shepherd 137Cs (30 y half life) irradiator (Shepherd and Associates, San Fernando, CA). After irradiation, dishes were incubated at 37°C in a humidified atmosphere of 95% air 5% CO2 for 24 h. TUNEL was performed as previously described [13].

Measurement of Apoptosis

CH-11-induced apoptosis was measured by flow cytometry to assess caspase-3 activity. CDDP- and IR-induced apoptosis was measured by TUNEL to determine DNA fragmentation.

TUNEL

This was performed as previously described [13] using the *in situ* cell death detection kit, POD (Roche, Indianapolis, IN). Briefly, cells were resuspended at a concentration of 1×10^6 cells/mL and 100,000 cells were placed onto microscope slides. They were allowed to dry at room temperature and then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. Antigen retrieval was achieved with 0.01 M sodium citrate (pH 6.0) in an 80°C water bath for 25 min. The slides were then washed in dH_20 for 5 min while shaking followed by a 5 min PBS wash while shaking. Endogenous peroxidase activity was quenched by incubating in 3% fresh hydrogen peroxide in methanol for 20 min while shaking. The slides were then washed three times for 5 min each in PBS while shaking. Blocking was performed with 50 μL of biotinylated link universal (Dako Cytomation, Carpinteria, CA) for 1 h at room temperature. The slides were then rinsed three times for 5 min in PBS while shaking and then reacted with a combination of 45 μL of label solution and 5 μL of enzyme solution for 1 h at 37°C. The cells were then rinsed five times for 5 min each in PBS and reacted with 50 μL of POD. The slides were then washed three times with PBS for 5 min each. The slides were then developed with DAB. Counterstaining was performed with hematoxylin for 20 s. The slides were then washed with H_2O and dehydrated with 70% ethanol for 5 min, followed by 90% ethanol for 5 min, 100% ethanol for 5 min, and xylene for 5 min. Slides were allowed to dry and mounted for