Sensitization of human colorectal cancer cells to paclitaxel-induced apoptosis by inhibition of MEK/ERK pathway is caspase-4 dependent

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Abstract: -Our previous studies have shown that paclitaxel induces the endoplasmic reticulum (ER) stress response in colorectal cancer (CRC) cells. Inhibition of MEK/ERK pathway sensitized CRC cells to paclitaxel-induced apoptosis by down-regulation of GRP78. In here, we report that induction of apoptosis by paclitaxel when GRP78 is down-regulated involves activation of caspase caspase-3, caspase-4, and caspase-9. Caspase-4 seemed to be the apical caspase in that caspase-4 activation occurred before activation of caspase-9 and caspase-3. Co-immunoprecipitation studies revealed that GRP78 is physically associated with caspase-4 before and after treatment with paclitaxel. Taken together, these results indicate that GRP78 might be a novel mechanism underlying resistance of CRC cells to microtubule-targeting drugs by binding to and inhibition of caspase-4. Combination of compounds capable of suppressing GRP78 might be a golden approach for improving the effectiveness of taxanes in treatment of CRC.

Key-Words: Colon Cancer, Paclitaxel, UPR, GRP78, Apoptosis.

1 Introduction

A number of cellular stress conditions, such as hypoxia, alterations in glycosylation status, and disturbances of calcium flux, lead to accumulation of unfolded and/or misfolded proteins in the endoplasmic reticulum (ER) lumen and cause so-called ER stress [1-3]. The ER responds to the stress conditions by activation of a range of stress-response signaling pathways that couple the ER protein folding load with the ER protein folding capacity and is termed the unfolded protein response (UPR) [1-3]. The UPR of mammalian cells is initiated by three ER transmembrane proteins-activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and doublestranded RNA-activated protein kinase-like ER kinase (PERK) [1–3]. Under unstressed conditions, the luminal domains of these sensors are occupied by the ER chaperon glucose-regulated protein 78 (GRP78) [1–3].
Fig. 1: UPR status in CRC cells. (A) GRP78 in a panel of CRC cell lines. Whole cell lysates were subjected to Western blot analysis. Western blot analysis of GAPDH levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. (B) RT-PCR products of XBP1 mRNA from CRC cells digested with ApaLI for 90 min followed by electrophoresis. The longer fragment derived from the active form of XBP1 mRNA and two shorter bands derived from the inactive form are indicated. Data are representative of three individual experiments. (C) SW480 cells were treated with paclitaxel (40µM) or TM (1 µM) for the indicated time periods. Whole cell lysates were subjected to Western blot analysis. Western blot analysis of GAPDH levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. (D) SW480 cells were treated with paclitaxel (40µM) for 16 hours. Whole cell lysates were subjected to Western blot analysis. Western blot analysis of GAPDH levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments.
Caspase-12 is thought to be a key mediator as caspase-12-deficient mouse cells are partially resistant to ER stress-induced apoptosis [4]. However, caspase-12 is expressed only in rodents. Its human homologue is silenced by several mutations during evolution [5]. Human caspase-4 has been shown to fulfill the function of caspase-12 and plays an important role in ER stress-induced apoptosis of human neuroblastoma and HeLa cells [6]. Moreover, the c-Jun NH2-terminal kinase (JNK), the transcription factor CAAT/enhancer binding protein homologous protein (CHOP), and deregulation of Bcl-2 and inhibitor of apoptosis protein family members have all been suggested to play roles in ER stress-induced apoptosis [7, 8, 9, 12].

There is increasing evidence to show that the UPR is activated in various solid tumors, perhaps due to nutrient deprivation and hypoxia [e.g., elevated expression of GRP78 has been reported in several cancers, such as breast cancer, melanoma, and prostate cancer [13–16]. Moreover, GRP78 expression has been shown in some cases to be associated with tumor development and growth and correlated with resistance to certain forms of chemotherapy. It seems that some cancer cells may have adapted to ER stress by activation of the UPR without resulting in apoptosis [14, 15, 17]. The central feature of this adaptive response has been suggested to be maintenance of expression of proteins that facilitate survival, such as GRP78 [18]. In addition, other survival signaling pathways, such as the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathways, may also play roles in counteracting the apoptosis-inducing potential of ER stress [13, 19].

2 Problem Formulation

Resistance of human CRC cells to the available chemotherapeutic agents is considered a major obstacle to the successful treatment. The purpose therefore of a new approach in treatment of CRC comes from the identification of the mechanisms employed in induction of apoptosis by chemotherapy and the possible resistance mechanisms in CRC cells against chemotherapy-induced apoptosis. In the present study we will examine the potential of the ER chaperon GRP78 in regulation of the sensitivity of CRC cells to paclitaxel-induced apoptosis. First we will evaluate the UPR status in a panel of CRC cell lines by measuring the constitutive levels of GRP78 and XBP-1 splicing in a panel of CRC cell lines using immunoblotting analyses and PCR [20]. Next we will study the apoptosis inducing potential of paclitaxel in CRC cells transfected with or without GRP78 siRNA pools [noor] and to study the correlation between levels of GRP78 and the potential of paclitaxel to induce apoptosis of CRC cells using the propidium iodide method [21].

3 Problem Solution

3.1 Expression of GRP78 in CRC Cell Lines

Constitutive activation of Ras/Raf/MEK/ERK signalling is a hallmark of many human cancers such as breast, lung, colorectal cancers, and melanoma [11, 22, 23]. ERK1/2 lies downstream of a group of kinases including protein kinase C (PKC), Raf-1, and MEK1. On stimulation by extracellular signals, they are successively activated by phosphorylation [24]. Previous studies have shown that MEK/ERK signaling pathway is associated with suppression of apoptosis [22, 25, 26]. Furthermore, activation of the ERK1/2 pathway has been found during ER stress, and governs cell survival during ER stress-induced apoptosis [11, 27]. Our previous studies have shown that paclitaxel induces the endoplasmic reticulum (ER) stress response in CRC cells. Inhibition of MEK/ERK pathway sensitized CRC cells to paclitaxel-induced apoptosis by down-regulation of GRP78 [20].
Fig. 2: (A) Down-regulation of GRP78 expression increases Paclitaxel-induced apoptosis of SW480. Cells were transfected with either a non-targeting siRNA (Control siRNA) or with a GRP78 specific siRNA sequence (Darmahcon) at 100nM for 24 hours. Transfected cells were then treated with paclitaxel for another 48 hours before harvest. Cells were analysed for apoptosis using the propidium iodide method. The data are representative of three individual experiments. (B) Correlation between the relative expression of GRP78 and the levels of paclitaxel-induced apoptosis in a panel of CRC cell lines. GRP78 relative expression was determined by dividing the densitometric value of the test protein by that of the GAPDH control. Regression analyses were carried out in a Macintosh computer using the StatView software.
Recently, it has been shown that the ER chaperone GRP78 expression contributes to ant apoptotic effects and chemotherapy resistance in many cancers [13, 16]. The levels of GRP78 were correlated well with ERK1/2 pathway activation in renal epithelial cells [28].

To study if CRC cells may express increased levels of GRP78, we examined GRP78 expression in a panel of CRC cell lines using immunoblotting analyses. Results showed that CRC cell lines expressed varying levels of GRP78 (Fig.1A). Furthermore, we evaluated the expression of another commonly used indicator of UPR activation, the spliced XBP1, by the PCR. Spliced XBP1 was observed in all CRC cells but to varying extents being the highest levels in HCT116 cells followed by SW620, HT29, and SW480 CRC cells and the least in Colo205 and WiDr cells (Fig.1B).

3.2 Down-regulation of GRP78 sensitized CRC cells to paclitaxel-induced apoptosis
We studied the importance of GRP78 in protecting CRC cells from paclitaxel-induced apoptosis by down-regulating GRP78 expression in CRC cells using small interfering RNA molecules. Treatment of SW480 cells with paclitaxel induced a time-dependent increase in the protein levels of GRP78 (Fig.1C). Tunicamycin (TM) at 3µM was used as a positive control. Similarly, treatment of a panel of CRC cell lines with paclitaxel resulted in varying degrees of increase in GRP78 protein levels among different CRC cells (Fig.1D). Downregulation of GRP78 levels significantly (p ≤ 0.05) sensitized SW480 cells to paclitaxel-induced apoptosis (Fig.2A). We next examined whether the variable expression of GRP78 levels is correlated with sensitivity to paclitaxel. Fig.2B indicates that the expression of GRP78 was inversely correlated with the degree of paclitaxel-induced apoptosis.

3.3 Sensitization of CRC cells to paclitaxel-induced apoptosis by down-regulation of GRP78 is caspase-dependent.
We examined if sensitization of CRC cells to paclitaxel-induced apoptosis by inhibition of GRP78 depends on the caspase cascade. After the down-regulation of GRP78 using siRNA, SW480 cells were treated with the general caspase inhibitor z-VAD-fmk 1 hour before the addition of paclitaxel for another 48 hours. Fig.3A shows that z-VAD-fmk markedly inhibited apoptosis induced by paclitaxel in cells where GRP78 was downregulated, suggesting that the caspase cascade plays a determining role in this sensitization.

To further elucidate the caspases involved, we studied the effects of specific inhibitors against caspase-3, z-DEVD-fmk, caspase-4, z-LEVD-fmk, caspase-8, z-IETD-fmk, and caspase-9, z-LEHD-fmk, on GRP78 down-regulation-mediated sensitization of SW480 cells to paclitaxel-induced apoptosis. As shown in Fig.3A, the inhibitor against caspase-4, caspase-9, or caspase-3 inhibited apoptosis induced by paclitaxel in the GRP78 knocked down SW480 cells to varying degrees. In contrast, the inhibitor against caspase-8 exhibited only minimal inhibitory effects (Fig.3A). These observations suggest that caspase-4, caspase-9, and caspase-3 are involved in sensitization of CRC cells to paclitaxel-induced apoptosis by GRP78 downregulation.

Activation of caspase-4, caspase-9, and caspase-3 in SW480 cells after down-regulating GRP78 using siRNA was also confirmed in fluorometric assays detecting activities of the caspases by specific substrates in whole cell lysates as shown in Fig.3B. Caspase-4 appeared to be activated first and to a higher degree compared to other caspases at 9 hours following treatment of SW480 cells with paclitaxel. Activated caspase-3 correlated well with activation of caspase-9. Activation of caspase-8 was delayed and became active after caspase-3 activation indicating that caspase-8 was unlikely to be an initiator of paclitaxel-induced apoptosis.
3.4 GRP78 is associated with caspase-4 and prevents its activation in CRC cells

How caspase-4 is activated by paclitaxel when the GRP78 is inhibited in CRC cells is not clear. In the murine system, several mechanisms have been suggested to be responsible for ER stress-induced caspase-12 activation [4, 8]. The protease calpain, on activation by calcium released from ER,
can activate caspase-12 [4]. In addition, caspase-12 has also been reported to be activated by a direct association with the ER stress transducer IRE1α and the adaptor protein TRAF2 [8]. Moreover, GRP78 was decreased and its up-regulation by ER stress was blocked by inhibition of MEK in CRC cells [20].

We studied if there is a physical association between caspase-4 and GRP78 by co-immunoprecipitation. As shown in Fig.3C, the endogenous caspase-4 could be co-immunoprecipitated with endogenous GRP78 as reported for caspase-12 in the murine system [29-31].

4 Conclusion
Taken together, these results indicate that inhibition of MEK/ERK sensitizes cultured human CRC cells to paclitaxel-induced apoptosis. This is mediated, at least in part, by activation of caspase-4, which is otherwise suppressed by the ER chaperone GRP78. These results suggest that GRP78 might be a novel mechanism underlying resistance of CRC cells to microtubule-targeting drugs by binding to and inhibition of caspase-4. Combination of compounds capable of suppressing GRP78 might be a golden approach for improving the effectiveness of taxanes in treatment of CRC.

References


