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ABSTRCT:

Magnolol has been reported to have anticancer activity. In this study we found that treatment with 100 £gM magnolol induced apoptosis in cultured human hepatoma (Hep G2) and colon cancer (COLO 205) cell lines but not in human untransformed gingival fibroblasts and human umbilical vein endothelial cells. Our investigation of apoptosis in Hep G2 cells showed a sequence of associated intracellular events that included (a) increased cytosolic-free Ca^{2+}; (b) increased translocation of cytochrome c (Cyto c) from mitochondria to cytosol; (c) activation of caspase-3, caspase-8, and caspase-9; and (d) downregulation of bcl-2 protein. Pretreatment of the cells with phospholipase C inhibitor, 1-[6-[[(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1 H-pyrrole-2,5-dione, or intracellular chelator of Ca^{2+}, 1,2-bis(2-aminophenoxy)ethane-N,N, N',N' -tetraacetic acid acetoxymethyl ester, inhibited the subsequent magnolol augmentation of $[Ca^{2+}]i$ and also the activation of caspase-8 and caspase-9, so that the occurrence of apoptosis in those cells was greatly reduced. Pretreatment of the cells with ZB4 (which disrupts the Fas response mechanism) also decreased the subsequent magnolol-induced caspase-8 activation and reduced the occurrence of apoptosis. We interpreted these findings to indicate that the above-listed sequence of intracellular events led to the apoptosis seen in Hep G2 cells and that [Ca2+]i , Cyto c, and Fas function as intracellular signals to coordinate those events.

Magnolol has been demonstrated to have anti-tumorigenesis effect against skin papilloma. The purposes of this project is to determined whether magnolol can (1) exert anti-cancer effect on GI tract malignancy in vitro (2) whether it involved

anti-proliferative effect or induction of apoptosis (3) what is mechanism of this anti-cancer action.

RESULTS

Magnolol Induces Apoptosis in Human Malignant Cell Lines

The occurrence of apoptosis, a natural death process in cells, most commonly is associated with arrested mitotic activity, decreased DNA replication, fragmentation of DNA, and activation of caspase-type enzymes. Accordingly, we examined COLO 205 and Hep G2 cells treated with magnolol to determine whether these indications of apoptosis appeared. At concentrations of 0±50 mM magnolol, apoptosis was not noted. When the magnolol concentration was increased to 100 mM, however, apoptosis was seen in COLO 205 (Figure 1a) and Hep G2 cells (Figure 1b) but not in cultured human nontransformed gingival fibroblasts (Figure 1c) and HUVEC (Figure 1d). Hence, magnolol was used at 100 mM in all of the remaining experiments in this study.

Magnolol Activates Caspase-3, Caspase-8, and Caspase-9

Because it has been suggested [17] that apoptosis requires the activation of caspases, we investigated the involvement of caspase activation in magnolol-induced apoptosis in Hep G2 cells by using Western blot analyses. The Hep G2 cells were grown in MEM containing 10% FCS and treated with 100 mM magnolol for various times, as indicated. Figure 2a shows that decreased staining in the procaspase-3 band and degradation of PARP, the substrate for caspase-3, were noted after 36 h of treatment with 100 mM magnolol, indicating that caspase-3 was activated. To elucidate the apoptotic pathways involved in the activation of caspase-3, we examined the changes of caspase-8 and caspase-9 protein levels in the magnolol-treated Hep G2 cells. After treatment of Hep G2 cells with 100 mM magnolol for 36 h, activation of caspase-8 and caspase-9 was evidenced by degradation of the pro-enzymes of caspase-8 and caspase-9 as well as by the appearance of their cleavage products (Figure 2a).

Magnolol Induces Cyto c Release From Mitochondria

It has been shown that activation of caspase-9 occurs during the release of Cyto c from mitochondria [18]. To examine whether this activation occurs in magnolol-induced apoptosis in Hep G2 cells, Cyto c release was monitored at various times after treatment with 100 mM magnolol. Figure 2b shows that magnolol treatment resulted in a significant accumulation of Cyto c in the cytosol fraction of cell extracts. This magnolol-induced elevation of cytosolic Cyto c was noted at 12 h

and peaked at 36 h after magnolol treatment. Under the same conditions, caspase activation was not seen until 36 h after magnolol treatment (Figure 2a). Evidently, translocation of Cyto c occurred in the magnolol-treated Hep G2 cells first, and activation of caspase-8 and caspase-9 and DNA fragmentation followed thereafter.

Magnolol Downregulates Bcl-2 Protein

Proteins of the bcl-2 family also are believed to be involved in the control of apoptosis [19]. Bcl-2 directly or indirectly operates to prevent the release of Cyto c from mitochondria. On the other hand, bax can trigger mitochondria to release Cyto c from mitochondria and thereby initiate apoptosis. Accordingly, we examined the changes of bcl-2 protein levels in magnolol-treated Hep G2 cells. Treatment of Hep G2 cells with 100 mM magnolol caused a time-dependent downregulation of bcl-2 protein, as shown by its decreased stain on gel (Figure 2c). In contrast, bax protein levels were not changed significantly.

Magnolol Elevates the Cytosolic-Free Ca^{2+} $[Ca^{2+}]i$ Concentration

Green and Reed [7] have proposed that the abnormal elevation of cytosolic-free Ca^{2+} is one of the major occurrences in apoptosis. Previously, Wang and Chen [3] reported that magnolol, applied in vitro to rat neutrophil cells, stimulates an increase in cytosolic-free Ca^{2+} in a dose-dependent manner. To examine whether magnolol-induced apoptosis is associated with an increase in cytosolic-free Ca^{2+}, we compared intracellular fura-2/AM fluorescence activity (an indicator of cytosolic-free Ca^{2+} concentration) in the Hep G2 cells treated with or without 100 mM magnolol. Figure 3a shows an initial rapid spike, with a sustained high $[Ca^{2+}]i$ curve in the magnolol-treated Hep G2 cells, which occurred rapidly and in a dose-dependent manner. Since elevation of $[Ca^{2+}]i$ can be caused by either Ca^{2+} influx from the external medium or by Ca^{2+}released from internal stores, we treated the cells with 2 mM EGTA to chelate the extracellular free Ca^{2+} .and prevent its entry into cells. Figure 3b shows that pretreatment with 2 mM EGTA for 100 s did not affect the magnitude of magnolol-induced elevation of cytosolic-free Ca^{2+}hence, the increase of $[Ca^{2+}]I$ is not due to an influx of extracellular Ca^{2+}. In contrast, pretreatment of Hep G2 cells with BAPTA/AM, an intracellular Ca^{2+} chelator, at 37C for 30 min reduced the magnitude of the magnolol-induced elevation of cytosolic-free Ca^{2+} (Figure 3c). Moreover, pretreatment of Hep G2 cells at 37C for 30 min with U73122 (phospholipase C inhibitor), which inhibits the hydrolysis of phosphatidylinositol biphosphate to inositol triphosphate [20], signi®cantly attenuated the magnolol-induced elevation of $[Ca^{2+}]i$ (Figure 3d).

Fas is Involved in Magnolol-Induced Caspase-8 Activation

It has been suggested that caspase-8 is associated with apoptosis involving a so-called death receptor [21]. Hep G2 cells express both Fas and Fas ligand [22]. Therefore, it is reasonable to speculate that Fas might be involved in caspase-8 activation in magnolol-treated Hep G2 cells. To address this issue, Hep G2 cells were treated with ZB4, the antibody antagonistic to Fas, which binds to the Fas ligand binding site on a Fas receptor and blocks Fas activation. We then examined DNA content frequency histograms, caspase levels, and caspase activity. Figure 4a-c shows representative DNA content frequency histograms from DMSO-, magnolol-, and ZB4+ magnolol-treated Hep G2 cells. In response to 100 mM magnolol treatment, the G_0/G_1 subpopulation, an indicator of apoptosis, was increased significantly (Figure 4b and d). We also found that pretreatment with ZB4 at a concentration of 250 ng/mL suppressed the magnolol-induced increase in the G_0/G_1 subpopulation (Figure 4c and d). It is clear that ZB4 partially reversed magnolol-induced apoptosis by 15±20%. ZB4 also inhibited magnolol-mediated caspase-8 activation, as evidenced by Western blot analysis and a caspase activity assay (Figure 4e). In contrast, pretreatment with ZB4 had no effect on magnolol-induced caspase-9 activation (Figure 4f).

Inositol Triphosphate±Mediated Pathway is Involved in Magnolol-Induced Caspase Activation

To study further the effect of elevated $[Ca^{2+}]_I$ on the development of apoptosis, Hep G2 cells were pretreated with 20 mM U73122 (phospholipase C inhibitor) for 30 min, followed by 100 mM magnolol treatment. Figure 5a-c shows representative DNA content frequency histograms from DMSO-, magnolol-, and U73122+ magnolol-treated Hep G2 cells. Figure 5c and d shows that pretreatment of these cells with U73122 for 30 min resulted in the inhibition of the magnolol-induced increase in the G_0/G_1 subpopulation. The graphs presented as Figure 5e and f show that magnolol-induced activation of caspase-8 and caspase-9 were inhibited substantially by pretreatment of Hep G2 cells with U73122. These findings suggested that the inositol triphosphate±mediated signaling pathway might be involved in the magnolol-induced increase of cytosolic-free Ca^{2+} which, in turn, led to apoptosis in Hep G2 cells through activation of caspase-8 and caspase-9.

DISCUSSION

A commonly held view is that uncontrolled cell proliferation in malignant tissues derives from a combination of two circumstances: increased cell multiplication unresponsive to normal control processes and decreased occurrence of the normal

process of cell death, that is, apoptosis. Our present state of knowledge, however, offers little insight regarding the intrinsic or extrinsic signals that might govern the opposing processes of cell multiplication and cell death to maintain normal cell populations. While we were engaged in our investigations of these matters, we discovered that the substance magnolol, derived from a herbal medication, has the potent effect of inducing apoptosis in two human cancer cell types (Hep G2 and COLO 205) but not in human nontransformed cells, such as gingival fibroblasts and HUVEC. In that initial part of our study, we found that even though magnolol, at the concentrations of 0-50 μ M, strongly inhibited growth and proliferation in these cells, it did not have a perceptible influence on apoptosis. While continuing this investigation, we found that magnolol at the higher concentration of 100 μM and employed in treatment periods of 36 h or more could indeed produce the characteristic signs of apoptosis. These findings show for the first time that magnolol can induce apoptosis in cultured cells.

Apoptosis is a cell-suicide mechanism that requires specialized cellular machinery. A central component of this machinery is a proteolytic system involving caspases, a highly conserved family of cysteine proteinases with speci®c substrates [17]. How caspases contribute to this process is not understood fully. It has been suggested that the terminal stages of apoptosis occur through the activation of caspases and that different initiator caspases mediate distinct sets of signals. By Western blot analysis, we showed that 100 mM magnolol induced activation of caspase-8 and caspase-9 in Hep G2 cells, as evidenced by decreases in stainable procaspase-8 and procaspase-9, and increases in caspase-8 and caspase-9 enzyme activities (Figure 4e and f). The active forms of caspase-8 and caspase-9 can cleave and activate downstream caspases, such as caspase-3 (Figure 2a), which eventually leads to apoptosis [23]. Caspase-9 is involved in death induced by cytotoxic agents [24,25]. Zou et al. postulated that Cyto c may activate caspases by binding to Apaf-1, which interacts with and activates caspase-9 [18]. Cyto c release sometimes can contribute to Fas-mediated apoptosis by amplifying the effects of caspase-8 to activate downstream caspases [26]. The active caspases, on the other hand, can promote Cyto c release and thereby amplify the signal for apoptosis [27]. In response to 100 mM magnolol, cytosolic Cyto c levels began to increase in about 12 h (Figure 2b), and significant activation of caspase-8 and caspase-9 occurred about 24 h later (Figure 2a). This finding suggested that the magnolol-mediated increase of cytosolic Cyto c could bring about activation of caspase-8 and caspase-9.

Although direct evidence of the involvement of bcl-2 and cytosolic-free Ca^{2+} in

magnolol-mediated Cyto c release is absent, the data of the present study suggested that downregulation of bcl-2 and elevation of cytosolic-free Ca^{2+} occurred during magnolol-mediated release of Cyto c. It has been suggested that bcl-2 family proteins are involved in the regulation of apoptosis through control of the release of Cyto c from mitochondria [19]. Bcl-2 prevents apoptosis by blocking the release of Cyto c from mitochondria [28]. Bax, on the other hand, directly induces Cyto c release from mitochondria and thereby triggers caspase-9 activation [29]. Treatment of isolated rat liver mitochondria with calcium chloride can trigger the release of Cyto c in vitro [30]. The results of the present study showed that elevation of the cytosolic-free Ca^{2+} concentration and decrease of the bcl-2 protein level induced by 100 μM magnolol treatment in Hep G2 cells seemed to be responsible for stimulating the release of Cyto c. The magnolol-mediated increase of cytosolic-free Ca^{2+} was released from the intracellular sources of calcium through inositol triphosphate-mediated pathways but not from Ca^{2+} influx across the plasma membrane. This result differs from that for magnolol-treated rat neutrophils, in which magnolol was found to stimulate Ca^{2+} release from internal stores and Ca^{2+} influx from extracellular sources across the plasma membrane [3]. The discrepancy between these two studies might be due to the differential effects of magnolol on varying cell types, such as liver cells versus neutrophils or cancer cells versus nontransformed cells.

The presence of the death receptor Fas and its ligand in Hep G2 cells is in accord with the hypothesis that caspase-8 is involved in magnolol-induced apoptosis in Hep G2 cells [22]. Administration of the anti-Fas antibody (ZB4) prevented caspase-8 activation (Figure 4e) and reduced magnolol-induced apoptosis by 15±20% (Figure 4d). Magnolol-mediated caspase-9 activation, however, was not affected by ZB4 treatment (Figure 4f). These results lend support to the idea that a Fas-mediated pathway is involved in magnolol-induced caspase-8 activation and consequent apoptosis in Hep G2 cells. It is noteworthy that surface expression of Fas was heterogeneous in malignant cell lines [31]. Therefore, it seems that activation of the Fas-mediated pathway does not always participate in magnolol-induced apoptosis. Whether magnolol activated Fas directly or promoted the action of a Fas ligand, which, in turn, activated Fas, remains to be determined.

Based on the results of the present study, we propose a model of the molecular mechanisms of magnolol-induced apoptosis in malignant cell lines, as shown in Figure 6. Although animal studies of magnolol-mediated anti-tumor action are ongoing, the findings from our previous study of magnolol's anti-cancer effect and the present in vitro studies strongly support the potential applications of magnolol in the

treatment of human cancer.

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As described in the preceding sections of this application, an important outcome of the proposed research will be a very detailed characterization of the molecular mechanisms regulating the apoptosis involved in the magnolol response in Hep G2 cells. The signal proteins involved in the apoptosis response to the cancer cells will be of great interest to design novel drug therapy strategy to hepatoma. These accomplishments will certainly represent a major step in furthering understanding of vitally important anti-tumorigenesis mechanism toward liver cancer.

Beyond the obvious benefit to be anticipated from the proposed research, it is hoped that this laboratory will offer many opportunities for a synergistic sharing of expertise and collaboration in research projects. Hopefully, we will be soon to develop effective method for treating patients with liver cancer diseases.

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