IG20, a MADD Splice Variant, Increases Cell Susceptibility to γ-Irradiation and Induces Soluble Mediators That Suppress Tumor Cell Growth

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ABSTRACT

The IG20 gene encodes at least four splice variants, including DENN-SV and IG20. DENN-SV is constitutively expressed at higher levels in tumor tissues. Cells transfected with the DENN-SV cDNA show increased resistance to tumor necrosis factor α (TNFα), TNF-related apoptosis-inducing ligand (TRAIL), etoposide, and vinblastine treatment, whereas overexpression of IG20 enhanced susceptibility to both intrinsic (drug) and extrinsic (e.g., TNFα and TRAIL) death signals. In this study, we investigated whether expression of the IG20 can render cells susceptible to γ-irradiation. Consistent with previous results, overexpression of DENN-SV and IG20 in HeLa cells conferred resistance and susceptibility, respectively, to the effects of γ-irradiation. HeLa IG20 cell susceptibility was attributable to enhanced apoptosis and reduced cell growth. This growth suppression was mediated by secreted soluble factors. Although HeLa DENN-SV cells grew more rapidly than control cells, replenishment with conditioned media from HeLa IG20 cells suppressed their growth. In addition, the conditioned media from HeLa IG20 cells stopped the growth of ovarian PA-1 cancer cells in the G1 -G0 cell cycle stage. Among an array of cytokines tested, interleukin 6 (IL-6) was found at the highest levels in HeLa IG20 culture supernatants, and IL-6 neutralization showed that it was, in part, responsible for the cell growth suppression. HeLa IG20 cells had elevated basal nuclear factor κB levels, a known regulator of IL-6 transcription. Finally, IG20 overexpression enhanced the combined apoptotic effects of TRAIL and γ-irradiation on HeLa cells. These results suggest that understanding further the mechanism of action of the IG20 splice variant may help in the advancement of cancer therapies.

INTRODUCTION

To date, we and others (1–4) have identified seven putative splice variants of the IG20 gene. The differences between the splice variants are limited to alternative splicing of exons 13L, 16, 21, 26, and 34. However, extensive analyses of expression of these splice variants using 57 human normal and tumor tissues and 14 different cell lines revealed that only four of the seven splice variants (namely IG20, MADD, IG20-SV2, and DENN-SV) are expressed. Interestingly, the IG20 gene is overexpressed in tumor tissues compared with normal tissues (2, 3). Additional examination of the gene expression patterns in normal and tumor tissues revealed that this overexpression was mostly represented by DENN-SV (4). When the IG20 variants were stably expressed in HeLa cells, it was observed that only two of the four variants could readily influence cell proliferation and induced cell death.

Overexpression of DENN-SV and IG20 in transfected cells conferred significant resistance and susceptibility, respectively, to tumor necrosis factor α (TNFα), and TNF-related apoptosis-inducing ligand (TRAIL) induced cell death by increasing caspase and nuclear factor κB (NFκB) activity, respectively. Furthermore, IG20 could potentiate TRAIL-induced apoptosis by increasing the recruitment of Fas-associatred death domain and caspase-8 to the death-inducing signaling complex.3

The contrasting effects of DENN-SV and IG20 on susceptibility to death-inducing stimuli suggest that the eventual outcome of these signaling pathways in tumor cells is determined, at least in part, by a balance in the expression levels of these two proteins. Indeed, HeLa cells that normally express all four variants on treatment with TNFα undergo apoptosis; however, approximately only one-half of the cells die. When dying cells were separated, on the basis of expression of apoptotic markers, from living cells and tested for expression of various splice variants, it was noted that whereas cells undergoing apoptosis expressed higher levels of IG20 (4), the viable cells expressed higher levels of DENN-SV.

The high level of expression of DENN-SV and the consequent imbalance in the relative levels of DENN-SV and IG20 found in human tumors suggest a role in tumor formation and/or propagation. It is already known that IG20 and DENN-SV can increase or decrease apoptosis, respectively, in stably transfected HeLa cells treated with etoposide or vinblastine (4). How IG20 and DENN-SV influence apoptosis activated by the intrinsic pathway remains unresolved. It is also not yet established whether these variants can similarly modulate the effects of γ-irradiation.

Radiation therapy takes advantage of the inherently unstable nature of tumors. The DNA lesions induced by γ-irradiation activate an intrinsic cellular pathway for dealing with DNA damage (5). Cells initiate a set of physiological responses thought to facilitate DNA repair processes that include cell cycle arrest in G1, S, and G2 (a slowing of DNA replication) phases and increased transcription of genes encoding proteins that participate in DNA repair and replication. If the degree of damage suffered by a cell is extensive, then the apoptotic pathway is activated, leading to cell death. At the molecular level, several pathways have been studied, including p53-dependent and p53-independent pathways (6, 7). Other molecules involved in the response to DNA damage include ataxia telangiectasia mutated, ataxia telangiectasia and Rad3 related, DNA-protein kinase, hCds1/Chk2, and p21. Although there is considerable insight on the molecular mechanisms that regulate DNA damage, the process remains unclear.

In this study, we examined the effects of IG20 on tumor cell growth and γ-irradiation-induced apoptosis. We demonstrate that the expression of IG20 enhances the effects of γ-irradiation, whereas DENN-SV enhances cell survival. We further demonstrate that HeLa cells stably transfected with IG20 produce soluble factors that can function in an autocrine or paracrine manner and markedly slow cell growth. We show that conditioned media (CM) from HeLa IG20 cells could cause growth arrest of PA-1 ovarian cancer cells, specifically at the G0–G1 stage of the cell cycle. Testing for a variety of soluble mediators showed a significant increase in interleukin 6 (IL-6) production by the HeLa IG20 cells. The induction of IL-6 was probably due to an increase in basal levels of NFκB activity. Neutralization of IL-6

Received 7/10/03; revised 10/13/03; accepted 10/20/03.

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3 M. Ramaswamy, E. V. Efimova, O. Martinez, N. U. Mulherkar, S. P. Singh, and B. S. Prabhakar. IG20 (MADD splice variant-5), a pro-apoptotic protein, interacts with DR4/DR5 and enhances TRAIL-induced apoptosis by increasing recruitment of FADD and caspase-8 to the DISC, submitted for publication.
showed that this cytokine was, at least in part, responsible for the observed effects of the CM from HeLa IG20 cells.

MATERIALS AND METHODS

Cell Culture. HeLa and PA-1 cells were grown in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS, 2 mm l-glutamine, and 100 units/ml penicillin/100 μg/ml streptomycin. To study the effects of CM, the regular growth medium was replaced with spent medium obtained from different cell cultures, as indicated under a given experiment.

γ-Irradiation. HeLa cells were harvested by trypsinization and irradiated in tubes. Cells were then plated at an initial density of 10⁴ cells/p100 plate and replenished with fresh culture medium. Cells were allowed to grow for 2 weeks, after which they were fixed with ice-cold methanol and stained with crystal violet to observe the outgrowth.

TRAIL Treatment. After γ-irradiation treatment, HeLa cells were plated in p100 plate at 10⁵ cells/plate. The next day, cells were treated in situ with 50 ng/ml of 1 ml/well TRAIL (Peprotech, Rocky Hill, NJ) for 3 h. Cells were then assayed for levels of active caspase-3 as an indicator of apoptosis.

Active Caspase-3 Assay. Cells were harvested, washed once with PBS, and then fixed in Cytofix/Cytoperm solution and washed with Perm/Wash buffer, as suggested by the manufacturer (PharMingen, San Diego, CA). Cells were then stained with phycoerythrin-conjugated rabbit anti-active caspase-3 antibodies according to the manufacturer’s protocol (PharMingen). The cells were then subjected to fluorescence-activated cell-sorting analysis to determine the percentage of cells positive for active caspase-3.

Staining for Mitotic Cells. PA-1 cells were plated (5 × 10⁴/well/p100). The next day, growth medium was replaced with either fresh medium or CM from control, HeLa IG20, or HeLa DENN-SV cells. Twenty-four hours later, cells were fixed with ice-cold methanol, washed three times, and stained with 4′,6-diamidino-2-phenylindole (1 μg/ml; Sigma Chemical Co., St. Louis, MO) to visualize mitotic cells.

Carboxyfluorescein Succinimidyl Ester (CFSE) Stain. Cells were stained with 2 μM CFSE (Molecular Probes, Eugene, OR) in situ [1 ml PBS/well (12 well plate)] for 10 min at 37°C, washed three times with PBS, and replenished with new media. Cells were harvested 48 or 72 h later and analyzed using a Becton Dickinson FACSCalibur equipped with CellQuest software. In the case of PA-1 cells, cells were stained in p100 plates as described above (5 × 10⁶ cells/plate).

Cell Cycle Staining. PA-1 cells (p100 plate) were harvested and washed in PBS three times. Cells were then treated with ice-cold 70% ethanol for 30 min on ice and washed with PBS plus 2% FCS two times and blocked for 10 min at room temperature with PBS plus 2% FCS. PBS containing propidium iodide (40 μg/ml; Sigma Chemical Co.) and RNase A (20 μg/ml; Roche, Indianapolis, IN) was used to resuspend washed cells. Finally, the cells were incubated at 37°C for 5 min before the analysis using a FACSCalibur. Data were analyzed using CellQuest and ModFit software.

Mpm2 Staining. Cells were harvested, washed once with PBS, and then fixed in Cytofix/Cytoperm solution and washed with Perm/Wash buffer, as suggested by the manufacturer (PharMingen). Cells were fixed with PBS containing 2% FCS at room temperature for 15 min and then stained with a mouse anti-mmp2 antibody (kindly provided by Igor Roninson, University of Illinois at Chicago, Chicago, IL) for 1 h and then washed twice in PBS plus 2% FCS. The mmp2 antibody recognizes a phosphorylated epitope (S/T) P found in phosphoproteins such as MAP2, HSP70, cdc25, and DNA topoisomerase II, most of which are phosphorylated at the onset of mitosis. The anti-Mmp2 binding was detected using an antimouse phycoerythrin-conjugated secondary antibody (1:100 dilution; Caltag, Burlingame, CA) for 1 h at room temperature on shaker. Wells were then washed three times with washing buffer using a vacuum manifold. Phycocerythrin-conjugated secondary antibodies were added to the appropriate wells and incubated for 45 min in the dark with constant shaking. Wells were washed twice, the assay buffer was added to each well, and samples were analyzed using the Bio-Plex suspension array system, which includes a fluorescence reader and Bio-Plex Manager analytical software (Bio-Rad Laboratories, Hercules, CA). Data analysis was performed using five-parametric curve fitting.

IL-6 ELISA. HeLa cells (5 × 10⁵) were plated in p100 Petri dishes in serum-free or serum-containing media. Four days later, supernatants (CM) were harvested, diluted (1:10), and assayed for the presence of IL-6 cytokine using a human IL-6 ELISA kit (BioSource International), as outlined by the manufacturer. HeLa IG20 cell CM contained 5 ng/ml IL-6 (data not shown).

IL-6 Neutralization. PA-1 cells were plated at 5 × 10⁵/12-well plate. The next day, media from the PA-1 cells were replaced with 1 ml of CM from HeLa IG20, HeLa DENN-SV, or control cells alone or along with an IL-6 neutralizing antibody (50 μg/ml; Biosource, Camarillo, CA). Forty-eight hours later, cells were harvested and counted.

RESULTS

We used previously characterized (1) HeLa cells stably transfected with a vector control, DENN-SV, or IG20. Equal numbers of cells were treated to 6, 8, 10, and 12 Grays of γ-irradiation, plated, and then allowed to grow for 2 weeks, after which they were stained with crystal violet. Transfected HeLa cells began responding differently at 8 Grays of irradiation, and this difference increased with the dose. A representative experiment (Fig. 1B) demonstrates the effect of 12 Grays of irradiation on each of the transfected cell lines. HeLa DENN-SV cells were highly resistant, whereas HeLa IG20 cells were more susceptible, to the effects of γ-irradiation compared with control cells. Very similar results were obtained when PA-1 ovarian carcinoma cells permanently transfected with IG20 were treated with γ-irradiation (4).

How IG20 renders cells more susceptible to the effects of γ-irradiation is unclear. First, we wanted to determine whether the effects of γ-irradiation on HeLa IG20 growth was primarily due to enhanced susceptibility or to reduced cell proliferation. IG20, DENN-SV, and control cells were exposed to different amounts of γ-irradiation, and then the percentage of cells undergoing apoptosis was assayed by measuring active caspase-3. As shown in Fig. 1C, there was a relatively small difference between the degrees of apoptosis seen after γ-irradiation in HeLa IG20 and DENN-SV cells, compared with controls (~10–14%). This indicated to us that the differences in the number of HeLa IG20 and DENN-SV colonies (Fig. 1B), relative to controls, cannot be accounted for by differences in apoptosis alone. Therefore, we evaluated the effects of IG20 and DENN-SV on the growth properties of HeLa cells.

Equal numbers of HeLa control, IG20, and DENN-SV cells were plated and then counted every other day for a total of 9 days. As shown in Fig. 2A, there was a significant difference in the numbers of

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Unpublished results.
cells over the 9-day period. The HeLa DENN-SV cell numbers were dramatically increased, whereas the number of HeLa IG20 cells was lower relative to controls.

Next, we wanted to confirm that the differences in cell numbers shown in Fig. 2 were because of differences in their growth rate and not because of differences in cell death. To determine the relative rate of cell division, the CFSE dye was used to stain the intracellular protein content of these cells. As the cells divide, the CFSE intensity decreases by half, which can then be assayed for by flow cytometry. As can be seen in Fig. 2B, there is a reduced dilution of CFSE in the HeLa IG20 cells compared with the control and HeLa DENN-SV cells. This indicated a lag in the division time of the HeLa IG20 cells compared with the other two cells and demonstrated that the differences in the numbers of cells seen in the growth curve is attributable, to a significant extent, to differences in the rate of cell division. These results show that IG20 renders cells more susceptible to the apoptotic effects of γ-irradiation and slows the rate of cell division.

Interestingly, the growth curve shown in Fig. 2A demonstrates a biphasic mode. Early on, all three cells demonstrated similar growth, but, subsequently, they showed divergence. This suggested that the effects on cell proliferation might depend on the accumulation of a critical factor(s) in the culture that either promotes (as in DENN-SV cells) or inhibits (as in IG20 cells) cell growth. To test this, we performed trans-well chamber experiments, which indicated that CM from IG20-transfected cells could suppress the growth of control cells as well as IG20- or DENN-SV-expressing vectors were subjected to 12 Gy of γ-irradiation. Cells were allowed to grow for 2 weeks, fixed and visualized for outgrowth. These results are representative of three experiments. The assay was also repeated using two different stably transfected HeLa populations. C, control HeLa, IG20, and DENN-SV cells were subjected to 6, 8, 10, and 12 Gy of γ-irradiation or were left untreated (Con). Cells were then plated at 10^6 cells/p100 plate. Twenty-four hours later, cells were assayed for the levels of active caspase-3. The results shown are representative of two experiments.

Fig. 1. Effect of IG20 and DENN-SV on outgrowth of HeLa cells after γ-irradiation. A, schematic representation of the splice variants of the full-length IG20 gene. Only four splice variants (IG20, MADD, DENN-SV, and IG20SV2) are readily seen in many normal and cancer tissues (4), and only IG20 and DENN-SV (large black arrows) modulate the death-inducing signals of tumor necrosis factor α (TNFα), TNF-related apoptosis-inducing ligand (TRAIL), and chemotherapeutic drugs. B, HeLa cells stably transfected with a control vector or DENN-SV- or IG20-expressing vectors were subjected to 12 Gy of γ-irradiation. Cells were allowed to grow for 2 weeks, fixed and visualized for outgrowth. These results are representative of three experiments. The assay was also repeated using two different stably transfected HeLa populations. C, control HeLa, IG20, and DENN-SV cells were subjected to 6, 8, 10, and 12 Gy of γ-irradiation or were left untreated (Con). Cells were then plated at 10^6 cells/p100 plate. Twenty-four hours later, cells were assayed for the levels of active caspase-3. The results shown are representative of two experiments.
The cells were allowed to grow for 3 more days (for a total of 7 days). Our results showed that untreated HeLa IG20 and HeLa DENN-SV cells had half and twice the number of cells seen in controls, respectively (Fig. 3A). HeLa DENN-SV cells treated with CM from HeLa IG20 cultures grew as slow as the HeLa IG20 cells (Fig. 3A). Replenishing HeLa IG20 cells with CM from HeLa DENN-SV cultures rescued cell numbers (Fig. 3A). It was not certain whether the reduction in the number of cells was attributable to cell death or differences in cell proliferation. To determine relative differences in the rate of cell replication, we stained the cells with CFSE and tested for dye dilution after treatment with different CM. Fig. 3B shows the cell division rates normally seen in control, DENN-SV, and IG20 cells. Figs. 3Bi and III show the effects of reciprocal exchange of CM between HeLa DENN-SV cells and HeLa IG20 cells. The addition of HeLa IG20 CM to HeLa DENN-SV cells reduced their rate of cell division (Fig. 3B), whereas replacement of HeLa IG20 medium with CM from HeLa DENN-SV alleviated the reduction in cell division.

It is apparent that the HeLa IG20 cells produce a soluble factor(s) that could suppress its own growth as well as that of other HeLa cells. Next, we tested to see whether this effect could be seen when cells unrelated to HeLa cells were exposed to the CM from HeLa IG20 cells. The CM from confluent HeLa IG20 cells, but not from control or DENN-SV cells, profoundly suppressed the cell growth of PA-1 ovarian cancer cells (Fig. 4A). This effect was again caused by a reduction in cell growth and not enhanced apoptosis, because these cells did not dilute their CFSE stain to the same extent as cells that were treated with CM from control or HeLa DENN-SV cells (Fig. 4B, I, II, and III).

Fig. 5 shows that mitotic bodies, as revealed by nuclear 4′,6-diamidino-2-phenylindole staining, are lacking in PA-1 cells treated with CM from HeLa IG20 cells. A total lack of PA-1 cell division was also corroborated using mpm2, a mitosis-specific antibody. The PA-1 cells left untreated or treated with CM from control HeLa, HeLa DENN-SV, or HeLa IG20 cells for 24 h were found to have 2.2, 2.9, 2.9, and 0% cells staining positive for mpm2, respectively. Cell cycle analysis (Fig. 6) showed that the PA-1 cells treated with CM from HeLa IG20 cells were growth arrested in the G1-G2 stage of the cell cycle.

To determine the identity of the soluble factor(s) produced by HeLa IG20 cells, we subjected culture supernatants from control and HeLa IG20 cells to a multiplex assay to detect the presence of a variety of cytokines (Table 1). There was a mild up-regulation of many of the cytokines in the supernatant of HeLa IG20 cells compared with the control cells, but IL-6 was significantly up-regulated (~20-fold). Next, we measured the levels of IL-6 in CM from all three cell cultures and found a marked increase in only CM from HeLa IG20 cells and not from the other two (Fig. 7A). Although, Fig. 7A shows the amounts of IL-6 produced from cells grown in serum-free media, the results are the same from cells grown in serum-containing media.

Because one of the more important transcription factors involved in the regulation of IL-6 is NFκB, we tested for the basal levels of NFκB activity relative to the other two cells (Fig. 7B). To determine the potential contribution of IL-6 to the growth-inhibiting property of the CM from HeLa IG20 cell culture, PA-1 cell growth was assayed in the presence of CM from HeLa IG20, HeLa DENN-SV, and control cells treated with and without an IL-6-neutralizing antibody. Fig. 8 shows that the PA-1 cell growth inhibition mediated by the CM from HeLa IG20 cells can be reversed by the addition of an IL-6-neutralizing antibody. However, the anti-IL-6
DISCUSSION

Of the four different splice variants encoded by the IG20 gene, only IG20 and DENN-SV show effects on cell proliferation and induced death (Fig. 1A). DENN-SV is highly expressed in tumor tissues relative to normal tissues, and its overexpression in various cell lines renders them resistant to TNFα, TRAIL-, etoposide-, and vinblastine-induced apoptosis and enhances their proliferation. In contrast, IG20 renders cells susceptible to the above treatments and suppresses cell proliferation.

Along with chemotherapy, radiation therapy remains one of the most important modalities of treatment for cancer. Therefore, we tested to see whether IL-6 alone can inhibit cell growth. Surprisingly, the addition of 5 ng/ml IL-6 had little or no effect on cell growth and indicated that although IL-6 is required, it is not sufficient for growth inhibition (data not shown). This demonstrated that the IL-6 was, at least in part, responsible for the cell growth-inhibitory effects of the CM from HeLa IG20 cells.

Next, we determined the effects of IG20 overexpression on the susceptibility of HeLa cells to TRAIL- and γ-radiation-induced cell death. HeLa cells either transfected with a control vector or a vector containing IG20 were exposed to γ-radiation and allowed to grow. Twenty-four hours later, these cells were treated with TRAIL for 3 h and subjected to flow cytometry to determine the levels of caspase-3 as an indicator of apoptosis. These results showed that IG20 HeLa cells were more susceptible to either γ-radiation or TRAIL alone relative to controls (Fig. 9). However, this difference was more profound when the cells were exposed to a combined treatment with γ-radiation and TRAIL (Fig. 9).

Fig. 3. Effects of conditioned media (CM) from HeLa cells stably transfected with IG20 spliced variants. A. HeLa IG20, HeLa DENN-SV, and control cells (105 cells/ml00 plate) were plated and allowed to grow for 7 days. HeLa DENN-SV and HeLa IG20 cells had their media replaced daily from day 4 to day 7 with CM derived from HeLa IG20 and HeLa DENN-SV cells, respectively. At day 7, all cells were harvested and counted. These results were confirmed by repeating the experiment at least three times. B. day 4 HeLa DENN-SV, HeLa IG20, and control cells were stained with CFSE and harvested on day 7 and analyzed for CFSE content. Day 4 HeLa DENN-SV (I) and HeLa IG20 (II) cells were loaded with CFSE (two plates per cell type). One plate from HeLa DENN-SV cells was replenished with CM from HeLa IG20 cells (II), and one plate from HeLa IG20 cells was replenished with CM from HeLa DENN-SV cells (III).

antibody had no effect on the growth patterns of the PA-1 cells grown in the presence or absence of CM from other cells. Subsequently, we tested to see whether IL-6 alone can inhibit cell growth. Surprisingly, the addition of 5 ng/ml IL-6 had little or no effect on cell growth and indicated that although IL-6 is required, it is not sufficient for growth inhibition (data not shown). This demonstrated that the IL-6 was, at least in part, responsible for the cell growth-inhibitory effects of the CM from HeLa IG20 cells.

Although HeLa IG20 cells were more susceptible to γ-radiation-induced apoptosis, it alone was not sufficient to explain the reduction in their outgrowth after γ-radiation. On closer examination of the growth characteristics, we discovered that the HeLa DENN-SV and HeLa IG20 cells grew faster and slower, respectively, than HeLa control cells (Fig. 2). The HeLa IG20 cell growth curve demonstrated a biphasic pattern in which the growth was very similar to that of HeLa controls and HeLa DENN-SV cells up to day 5 and then abruptly changed with HeLa IG20 cells growing considerably slower. This suggested that a minimum concentration of a critical factor or signal might be required for the growth-suppressive effect. Trans-well experiments and reciprocal exchange of CM from IG20- and DENN-SV-transfected cells showed that a critical factor(s) was present in the culture supernatant of HeLa IG20 cells and that it could affect the growth of control and HeLa DENN-SV cells. Moreover, it also suggested that the growth-inhibitory property of the CM from HeLa IG20 cells could dominate the growth-potentiating properties of DENN-SV (Fig. 3BII).

The CM from HeLa IG20 cells had a profound effect on the ovarian cancer cell PA-1 and completely stopped its growth, as determined by cell proliferation (Fig. 4A), dilution of CFSE (Fig. 4B), staining for mitotic bodies (Fig. 5), and cell cycle analysis (Fig. 6). This clearly demonstrated that the soluble factor present in HeLa IG20 culture supernatant could not only suppress HeLa cell growth but also the growth of PA-1 ovarian cancer cells. Moreover, accumulation of PA-1 cells in the G0-G1 phase of the cell cycle suggests that the CM either induced PA-1 cells to undergo senescence (G0) or cell cycle arrest at the G1 checkpoint.

When supernatants from HeLa IG20 cells and control cells were compared for the amounts of several cytokines, we found some increase in the amounts of most of the cytokines tested, however, there was a profound increase in the amount of IL-6. This was confirmed further by our ELISA results that showed elevated levels of...
IL-6 in the CM from HeLa IG20 cells but not from the others. The importance of IL-6 in suppressing the growth of PA-1 cells was established further when a considerable reversal of the effect was noted in the presence of a neutralizing IL-6 antibody (Fig. 8). However, the antibody did not completely reverse the growth-suppressive effects of the HeLa IG20 CM and, moreover, the addition of IL-6 to growth medium did not suppress cell growth and suggested that IL-6 is required, but not sufficient, to cause growth suppression.

Indeed, it is not uncommon to see the production of IL-6 by tumor tissues and cancer cell lines, including cervical (8, 9) and ovarian cancers.
factor/H9260 fibroblasts could inhibit the growth of early melanomas but not. In a previous study, it was observed that IL-6 produced by skin ent kinase inhibitors (14, 15, 17). Differentiation is unclear but may involve the induction of cyclin-dependent cell cycle. The mechanism by which IL-6 induces growth arrest and/or. HeLa IG20, but not HeLa DENN-SV cells. A, 10^5 of three experiments. These results are representative. Four days later, supernatants were assayed for the presence of IL-6. Consistent with our results, previous studies have shown that on IL-6 treatment, early-stage melanoma cell lines (14), human prostate cell line LNCaP (15), and leukemic myeloblastic cells (16) are also growth arrested at the G1-G0 stage of the cell cycle. The mechanism by which IL-6 induces growth arrest and/or differentiation is unclear but may involve the induction of cyclin-dependent kinase inhibitors (14, 15, 17).

IL-6 production can also play a pivotal role in cancer progression. In a previous study, it was observed that IL-6 produced by skin fibroblasts could inhibit the growth of early melanomas but not advanced stage melanomas (18). This was not because of differences in the levels of expression of either the IL-6 receptor or the IL-6 transducer (gp130). Interestingly, the melanoma cells themselves secreted IL-6. Additional studies then showed that IL-6 undergoes transition from being a paracrine growth inhibitor to an autocrine stimulator (19) during human melanoma progression. These studies show the underlying complexities associated with the production of IL-6 by cancer cells and its autocrine/paracrine effects on them. Additional studies are needed to fully delineate the regulation and function of IL-6 in cancer cells. Nevertheless, our results clearly show that IG20 can significantly enhance the production of IL-6 by HeLa cells.

Several transcription factors are involved in the regulation of IL-6 production, including AP-1, NFκB, and nuclear factor IL-6 (20–23). However, it is likely that NFκB plays a prominent role in the enhanced production of IL-6 by HeLa IG20 cells. This is consistent with our finding that higher basal levels of active NFκB were found in HeLa IG20 cells compared with controls and HeLa DENN-SV cells (Fig. 7B).

From our previous studies, we know that TNFα can induce IG20 binding to TNF receptor 1 through its death domain, and it is well known that TNF receptor 1 can produce signals leading to the activation of NFκB. However, higher basal levels of active NFκB seen in HeLa IG20 cells are induced, whereas IG20 protein is not associated.

### Table 1 Cytokine concentrations in the supernatants of HeLa control and HeLa IG20 cells

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^IL, interleukin; ND, cytokines not detected; GM-CSF, granulocyte macrophage colony-stimulating factor; TNF, tumor necrosis factor; RANTES, Regulated upon Activation, Normal T-expressed and Secreted; MIP, macrophage inflammatory protein; bFGF, basic fibroblast growth factor; G-CSF, granulocyte colony stimulating factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor.

Fig. 6. The effect of conditioned media (CM) from HeLa IG20 cells on the cell cycle progression of PA-1 cells. PA-1 cells were plated in three p100 plates (5 × 10^3 cells/plate). Control cells were harvested 24 h later and analyzed for cell cycle progression (1). At this point, media from remaining plates were replaced with CM from either HeLa control (2) or HeLa IG20 (3). Forty-eight hours later, cells were subjected to cell cycle analysis.

Fig. 7. Interleukin 6 (IL-6) secretion and nuclear factor κB (NFκB) basal activity is up-regulated in HeLa IG20, but not HeLa DENN-SV cells. A, 10^5 HeLa vector, HeLa DENN-SV and HeLa IG20 cells were plated in p100 plates in serum-free media. Four days later, supernatants were assayed for the presence of IL-6. These are representative results from three experiments. B, HeLa vector and HeLa DENN-SV and HeLa IG20 cells were cotransfected with a reporter nuclear factor κB luciferase plasmid and a renilla luciferase plasmid driven by a cytomegalovirus promoter. Forty-eight hours later, cells were harvested, lysed, and assayed for firefly luciferase activity. These results are representative of three experiments.
with the TNFα receptor (1); therefore it remains unclear as to how expression of IG20 leads to basal NFκB activation, and additional studies are required to elucidate the mechanism.

IG20 can enhance radiation-induced apoptosis (Fig. 1C). This, coupled with our previous observations that IG20 can enhance TRAIL- and TNFα-induced apoptosis, suggests a convergence (24) of the extrinsic (i.e., TNFα and TRAIL) and the intrinsic (i.e., γ-irradiation) apoptotic pathways. Induction of the extrinsic pathway through TNFα and TRAIL binding to their cognate receptors activates caspase-8 and subsequently the effector caspase-3. When the cells are stressed, the intrinsic pathway is initiated through the mitochondria, resulting in the activation of caspase-9 and then caspase-3. Most likely, it is the enhanced caspase-3 activation that is the common event in the IG20-increased cell death mediated through both pathways. Alternatively, DNA damage caused by ionizing radiation or chemotherapeutic drugs can lead to death receptor up-regulation (25). An increase in the cell surface expression can cause receptor oligomerization, leading to signaling, including the formation of the death-inducing signal complex (24). If this were the case, then it is possible that IG20 may be enhancing radiation-induced cell death by increasing TNF receptor 1/death receptor 4/death receptor 5-mediated activation of caspase-8. Collectively, our results show that although increasing TNF receptor 1/death receptor 4/death receptor 5-mediated apoptotic pathways is of considerable interest because it holds promise for treating cancers that are resistant to conventional therapy. For example, it has been demonstrated (26) that the combined effects of TRAIL and etoposide or cis-diaminedichloroplatinum(II) (chemotherapeutic DNA-damaging agent) could cooperatively induce apoptosis of glioma cells in vitro and reduce tumor loads in nude mice in vivo. Studies are already underway to further characterize the mechanism of action of the combined therapy (27).

Our observations have significant implications for cancer therapy, because we have shown that DENN-SV is highly expressed in tumors with little or no expression of IG20. Furthermore, we showed that overexpression of IG20 can render PA-1 cells more susceptible to TRAIL-induced apoptosis.7 HeLa cells normally express both the antiapoptotic (DENN-SV) and the proapoptotic (IG20) variants (1). In this study, we found that the efficacy of γ-irradiation-induced cell death is enhanced by IG20 overexpression. Exposure to both TRAIL and γ-irradiation can work cooperatively and enhance apoptosis even more significantly (Fig. 9).

Collectively, our results show that IG20 can render cells more susceptible to apoptosis and suppress cell growth. This raises the possibility of using IG20 to render cells that are otherwise resistant to become more susceptible to various modalities of cancer therapy.

REFERENCES


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IG20, a MADD Splice Variant, Increases Cell Susceptibility to \(\gamma\)-Irradiation and Induces Soluble Mediators That Suppress Tumor Cell Growth

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