Membrane Rafts Segregate Pro- from Anti-Apoptotic Insulin-Like Growth Factor-I Receptor Signaling in Colon Carcinoma Cells Stimulated by Members of the Tumor Necrosis Factor Superfamily

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In the tumor microenvironment, autocrine/paracrine loops of insulin-like growth factors (IGFs) contribute to cancer cell survival. However, we report here that IGF-I can send contradictory signals that interfere with cell death induced by different ligands of the tumor necrosis factor (TNF) superfamily. IGF-I protected human colon carcinoma cells from TNF- α -induced apoptosis, but it enhanced the apoptotic response to anti-Fas antibody and TNF-related apoptosis inducing ligand stimulation. This proapoptotic effect of IGF-I, observed in several but not all tested colon cancer cell lines, was mediated via the phosphatidylinositol 3'-kinase (PI3K)/Akt pathway. Furthermore, IGF-I receptors (IGF-IR) were located in and out of membrane lipid rafts and were tyrosine autophosphorylated in response to IGF-I. However, disruption of rafts by acute cholesterol depletion shifted IGF-IR to non-raft domains, abolished the IGF-I-mediated proapoptotic effect, and inhibited the IGF-I-dependent IRS-1 and Akt recruitment into and phosphorylation/activation within lipid rafts. Replenishing cell membranes with cholesterol reversed these effects. Activation of extracellular-regulated kinase-1/2 and p38 mitogen-activated protein kinase, which convey the IGF-I anti-apoptotic effect, occurred independently of lipid rafts. Thus, we propose that segregation of IGF-IR in and out of lipid rafts may dynamically regulate the pro- and anti-apoptotic effects of IGF-I on apoptosis induced by TNF superfamily members. (*Am J Pathol* 2005, 167:761–773)

Apoptosis (programmed cell death) is a basic function that alongside proliferation and differentiation, is part of the repertoire available to the cell to respond to internal and external stimuli.¹ Dysregulation of apoptosis is linked to a variety of human diseases,² and resistance to apoptosis is a major hallmark of cancer cells.³ Failures in apoptosis indeed contribute to carcinogenesis by allowing survival of cells with genomic lesions and by promoting cell resistance to immune-based destruction. Moreover, resistance of cancer cells to apoptosis is of major concern in cancer therapy because apoptosis is the main mechanism whereby drugs, radiation, and immune cells induce the destruction of tumor cells.⁴ To acquire resistance to apoptosis, cancer cells use various strategies to interfere with critical control points in the cell death pathway. Numerous examples of down-regulation or mutation of proapoptotic genes and/or overexpression of anti-apoptotic genes have been reported in the literature.^{1–4} In addition, the importance of the cancer cell microenvironment in driving tumor progression has been recently emphasized.⁵ Thus, many growth factors, cytokines, and chemokines issued from the tumor stroma cells and the cancer cells themselves may establish multiple neo-regulatory networks that contribute to tumor growth, invasion, and metastasis.

Among the growth factors, the insulin-like growth factor (IGF) signaling system plays a prominent role in cancer development and progression.^{6–10} The IGF system comprises two ligands (IGF-I and IGF-II), the IGF-I cell surface receptor (IGF-IR) that transduces the biological signals from both IGFs, and a family of IGF-binding proteins (IGFBPs) that regulates IGFs bioavailability to receptors.¹¹ Ligand binding to the extracellular α -subunits of

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IGF-IR results in activation of the intrinsic tyrosine kinase within the intracellular part of the IGF-IR β -subunit, which induces autophosphorylation and leads to recruitment and tyrosine-specific phosphorylation of several substrates. The insulin receptor substrate-1 (IRS-1) and Src homology collagen (Shc) are the best characterized docking proteins. These proteins can then bind SH2-containing proteins, which next results in stimulation of an array of intracellular signaling cascades. Among them, the phosphatidylinositol 3'-kinase (PI3K)/Akt (also known as protein kinase B) pathway and the different sets of mitogen-activated protein kinase (MAPK) pathways are the major signal transduction cascades that ultimately trigger multiple biological cell responses to IGFs.^{6-8,12,13}

It is now well-documented that the transforming activity of IGF-IR depends, to a large extent, on its potent anti-apoptotic activity against a wide variety of proapoptotic stimuli.^{6-8,12} By using as a model the p53-deficient HT29-D4 human colon carcinoma cell line, we reported that IGFs induced a strong resistance against apoptosis induced by tumor-necrosis factor- α (TNF) in interferon- γ (IFN)-sensitized cells. The anti-apoptotic activity of the activated IGF-IR was mediated through its ability to potentiate TNF receptor 1 (TNFR1)-induced extracellular signal-regulated kinase (Erk)-1/2 and p38 MAPK, and nuclear factor (NF)- κ B signaling pathways. In contrast, activation of the PI3K/Akt pathway was not required for IGF-IR to induce resistance against IFN/TNF-induced apoptosis.^{14,15}

Ligands of the tumor-necrosis factor superfamily (TNFSF) are essential cytokines that exert a lot of biological functions primarily, but not exclusively, within the immune system. Some of them, such as TNF, Fas ligand (FasL, also called APO-1L or CD95L), and TNF-related apoptosis-inducing ligand (TRAIL, also called APO-2L) are expressed at the surface of natural killer cells and cytotoxic T lymphocytes and can mediate via their cognate death receptors (DRs) apoptosis of oncogenically transformed cells.^{3,4,16,17} However, signaling through TNFR1 is quite different from signaling through Fas or TRAIL DR (TRAIL-R1/DR4 and TRAIL-R2/DR5). These latter DRs indeed deliver a powerful proapoptotic signal whereas TNFR1 signaling is dual and may induce cell death but also promote cell survival, inflammation, and cell migration.^{16,17} Thus, in parallel with its potential capability to initiate a tumor apoptotic response, TNF can also facilitate tumorigenesis,¹⁸ and IGF-I can exacerbate the pathways that support this protumorigenic function.^{14,15} This paradoxical role of TNF prompted us to re-evaluate the IGF-I-mediated regulatory functions on apoptosis induced by different members of the TNFSF in colon cancer cells.

In this study, we report that IGF-I can either exert a strong anti-apoptotic effect on TNF-induced apoptosis or surprisingly enhance apoptosis induced by anti-Fas or TRAIL stimulation in several human colon cancer cell lines. Remarkably, the IGF-I proapoptotic effect appears to be mediated via activation of the PI3K/Akt pathway within cholesterol-rich membrane microdomains, known as lipid rafts. In contrast, activation of Erk 1/2 and p38

MAPK that convey the IGF-I anti-apoptotic signaling occurs out of rafts. Thus, segregation of IGF-IR in and out of lipid rafts may contribute to its contradictory regulatory effect on TNF-induced versus FasL- and TRAIL-induced colon cancer cell death.

Materials and Methods

Reagents

Unless noted, all reagents were purchased from Sigma-Aldrich (L'Isle d'Abeau, France). Recombinant human des-(1-3)-IGF-I (dIGF-I) was obtained from Gro-Pep (Adelaide, Australia). IFN, TNF, and TRAIL were from Peprotech, London, UK. Anti-Fas IgM monoclonal antibody (mAb) (7C11) was obtained from Beckman-Coulter, Fullerton, CA. Rabbit polyclonal Abs raised against phospho (p)-Akt (Ser-473), Akt (1-3), p-Erk 1/2 (Thr-202/Tyr-204), and p-p38 (Thr-180/Tyr-182) were purchased from Cell Signaling, Beverly, MA. Rabbit polyclonal Abs raised against p38 (C-20), Erk-1/2 (K-23), caveolin 1 (N-20), IRS-1 (H-165), IGF-IR (β -subunit; C-20), and mouse mAb raised against clathrin heavy chain (TD.1) and p-Tyr (PY-99) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG secondary Abs, and enhanced chemiluminescence reagents were from Amersham Biosciences, Buckinghamshire, UK. Pharmacological inhibitors were from Alexis Biochemicals, San Diego, CA (PD098059, LY294002, SB203580) and BioMol, Plymouth Meeting, PA (triptolide). YO-PRO-1 fluorescent dye was from Molecular Probes, Eugene, OR.

Cell Lines and Cell Culture

Nine human colon carcinoma cell lines were used in this study. HT29-D4 cells were cloned by limiting dilution of the HT29 parental cell line.¹⁴ HRT-18 cells were purchased from American Type Culture Collection (Manassas, VA) and HCT-116 cells were purchased from European Collection of Cell Cultures (Salisbury, UK). HCT-15 and COLO 205 cells were kindly provided by Dr. F. Montero-Julian (Immunotech, Marseille, France). SW 480, SW 620, HCT-8/E11, and HCT-8/E11R1 cells, these latter being α -catenin-deficient,¹⁹ were a generous gift of Pr. M. Mareel (Gent University Hospital, Gent, Belgium). Each cell line was routinely cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (both from Life Technologies, Grand Island, NY) at 37°C in 5% CO₂.

Cell Death Assays

Cells were seeded at 1.5×10^5 cells/cm² in fetal calf serum-containing Dulbecco's modified Eagle's medium and grown to 80% confluence, then washed three times and further incubated for 24 hours in fetal calf serum-free Dulbecco's modified Eagle's medium supplemented with 0.1% fatty acid-free bovine serum albumin (SFM). To induce cell death, cells in SFM were incubated with or

without 40 ng/ml IFN for 15 minutes at 37°C, then washed twice, and treated with TNF (50 ng/ml), TRAIL (100 ng/ml), or anti-Fas mAb (100 ng/ml) in the presence or the absence of various concentrations of dIGF-I. dIGF-I is a NH2 terminally truncated analog that was used instead of IGF-I to avoid interfering effects with cell secreted IGFBPs.¹⁵ After a 24-hour period of incubation, YO-PRO-1 dye was added at a final concentration of 4.0 μ mol/L and incubated 2 hours at 37°C, then fluorescence was assayed on a fluorescent plate reader (Fluoroskan Ascent FL; Labsystems, Helsinki, Finland). Alternatively, quantitative analysis of DNA fragmentation, a hallmark of apoptotic cells,^{1,5} was done using a cell death detection enzyme-linked immunosorbent assay kit (Roche Diagnostics GmbH, Mannheim, Germany) by measuring relative amounts of DNA-histone complexes released in the cytoplasm. DNA fragmentation was also analyzed by flow cytometry. Briefly, nonadherent and adherent cells were harvested, combined, and treated by a fluorochrome hypotonic solution containing 50 μ g/ml propidium iodide in 0.1% sodium citrate and 0.1% Triton X-100, then apoptotic cell population was determined by flow cytometry for quantifying sub-G₁ content as previously described.¹⁴ Cell analyzing was performed on a Becton-Dickinson FacSort flow cytometer (San Jose, CA). Unless otherwise indicated, values are the mean $\pm$ SEM from four independent experiments made in duplicate. For experiments using pharmacological inhibitors, cells were incubated in SFM with 100 μ mol/L PD098059, 20 μ mol/L LY294002, or 20 μ mol/L SB203580 2 hours previously, or with 55 nmol/L triptolide (PG490) 5 hours previously, and throughout the cell death assay. Efficiency of inhibitors was systematically verified as previously reported.^{14,15}

RNA Isolation and cDNA Expression Arrays

Total cell RNA was isolated using RNAqueous-4PCR kit (Ambion Inc., Austin, TX) according to the manufacturer's recommendations, and double-stranded ³²P-labeled DNA probes were synthesized from 2 μ g of total RNA with the Strip-EZ RT kit (Ambion) using oligodT primers and according to the manufacturer's recommendations, then 10⁶ cpm per membrane were simultaneously hybridized to three pairs of DNA array membranes. The following GEArray membranes, Q series, were purchased from Superarray Bioscience Corporation (Frederick, MD): human apoptosis(HS002); human NF- κ B signal transduction(HS016); human signal transduction pathway (HS008). Lists of the 249 tested genes are available on Superarray's web site at www.superarray.com. All procedures were performed according to the recommendations of the GEArray User Manual. Membranes were exposed in a FLA-2000 Fuji phosphorimager and the labeled spots were quantified using Scanalyse (Eisen Laboratory, Berkeley, CA) and GEArray Analyser (Superarray) softwares. Membrane stripping was performed using the Strip-EZ RT kit protocol. To identify differentially expressed genes, the variation of the signal (dIGF-I plus TNF:TNF or dIGF-I plus TRAIL:TRAIL) in IFN-presensitized cells expressed in percentage was taken from the data of four independent assays after standardization

using the membrane housekeeping gene pool. Statistical significance was determined using the nonparametric Mann-Whitney test. Only the genes, the variation of which had $P < 0.05$, are presented.

Membrane Raft Preparation

Rafts were isolated by extraction with Brij 98 at 37°C followed by Optiprep density gradient centrifugation essentially as previously reported.²⁰ In brief, $\sim 30 \times 10^6$ cells were harvested with a rubber policeman and gently sonicated (five 5-second bursts, 5 W) in 1 ml of ice cold buffer A (25 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L EGTA, 10 mmol/L NaF, 5 mmol/L Na₃VO₄, 10 mmol/L NaP-P, and a mixture of protease inhibitors). The post-nuclear supernatant was recovered after centrifugation at $800 \times g$ at 4°C for 10 minutes, and then extracted with buffer A containing 1% Brij 98 for 5 minutes at 37°C, then diluted with Optiprep 60% containing 5% sucrose (final concentration: Optiprep, 43%; sucrose, 3.3%; Brij 98, 0.3%) and chilled down on ice for 1 hour before being placed on the bottom of a centrifuge tube. The samples were then overlaid with 1 ml of each 36%, 34%, 32%, 30%, 27.5%, 25%, 20%, and 0% Optiprep in buffer A. The gradients were spun at $175,000 \times g$ in a SW41 rotor (Beckman Instruments) for 16 hours at 4°C. Nine fractions were collected from top to bottom of centrifuge tubes and analyzed by Western blot.

Alternatively, the Brij 98-insoluble raft fraction was prepared as follows: cells were harvested and extracted with 1% Brij 98 at 37°C as described above, then spun at $800 \times g$ for 10 minutes. The postnuclear supernatant was centrifuged at $100,000 \times g$ for 60 minutes at 4°C and the supernatant referred as the soluble fraction (S) containing solubilized membrane and cytosolic fractions. The membrane raft fraction in the pellet (R) was resuspended in buffer A containing 1% Brij 98, 0.3% deoxycholic acid, and 60 mmol/L *n*-octyl- β -D-glucopyranoside (ODG), and nonsoluble material was removed by an additional centrifugation.

Immunoprecipitation and Western Blot Analysis

Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed with 20 mmol/L Tris-HCl (pH 8.0), 200 mmol/L NaCl, 1 mmol/L EDTA, either 1.0% Triton X-100 or 0.3% deoxycholic acid plus 60 mmol/L ODG as indicated under the figures, 10 mmol/L Na₃VO₄, 10 mmol/L NaP-P, 10 mmol/L NaF and a mixture of protease inhibitors. For immunoprecipitation, the lysate was incubated with 1.0 to 2.0 μ g of Ab overnight at 4°C, then protein G-conjugated agarose beads were added for 45 minutes at 4°C with constant stirring. The beads were then washed three times with lysis buffer, three times with lysis buffer containing 500 mmol/L NaCl, and once with PBS. Proteins extracted from equal amounts of cells within each experiment were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7 to 17% acrylamide), then electrophoretically transferred onto Hybond-C extra nitrocellulose sheets (Amersham) and

probed with the indicated primary Ab or HRP-coupled cholera toxin B chain. Bound Abs were then detected according to the enhanced chemiluminescence protocol and reagents from Amersham using HRP-conjugated anti-IgG as secondary Abs. Blots were quantified by scanning densitometry and absorbance curves integrated using the ImageMaster software (Amersham).

Cholesterol Depletion and Repletion Treatments

To analyze the effects of cholesterol depletion, cells were incubated in SFM with or without 10 mmol/L methyl- β -cyclodextrin (Me- β -CD), 10 mmol/L α -cyclodextrin (α -CD), or 1.0 U/ml cholesterol oxidase for 30 minutes at 37°C. To examine the effects of cholesterol repletion, cholesterol depletion was first performed as above with 10 mmol/L Me- β -CD for 30 minutes, then the cells were washed three times with SFM and thereafter incubated in SFM with 0.5 mmol/L cholesterol/Me- β -CD complexes for 120 minutes at 37°C to restore the membrane cholesterol level.²¹ Cholesterol/Me- β -CD complexes were prepared as described previously.²² After either cholesterol depletion or depletion/repletion, the cells were washed three times, and then prepared for the various assays as described above.

Cholesterol Assay

Pelleted membranes were subjected to lipid extraction with chloroform/methanol (2:1; v/v), and the cholesterol content was quantified by an enzymatic assay using cholesterol oxidase (Biomerieux, Marcy l'Etoile, France).

Results

IGF-I Sends Contradictory Signals in Controlling Apoptosis Induced by TNFSF Ligands

As shown in Figure 1A, HT29-D4 cells were totally resistant to cell death induced by TNF unless they were pre-sensitized by a 15-minute pulse with IFN. In IFN-sensitized cells, cell death induced by TNF and measured by the uptake of YO-PRO-1 dye was inhibited by dIGF-I in a dose-dependent manner with a maximal effect, ie, ~75% inhibition, for 50 ng/ml of dIGF-I (Figure 1A). These results are in complete agreement with our previously reported data.¹⁴

In contrast, HT29-D4 cells were susceptible to anti-Fas- and TRAIL-induced cell death, and pretreatment with IFN enhanced cell killing (Figure 1, B and C). Moreover, exposure of cells to dIGF-I induced an unexpected enhancement of cell killing triggered by either anti-Fas (Figure 1B) or TRAIL (Figure 1C) whether cells were pretreated or not with IFN. This dIGF-I's enhancing effect was dose-dependent and reached an optimum for 10 ng/ml, which induced an approximately twofold increase in cell death. At this latter concentration, dIGF-I did not bind to insulin receptor (IR) at the HT29-D4 cell surface,²³

Figure 1. IGF-I sends contradictory signals controlling TNFSF ligand-induced cell death in HT29-D4 cells. HT29-D4 cells were either left untreated (dashed line) or pulse-treated for 15 minutes with IFN (solid line), then incubated for 24 hours with TNF (A), anti-Fas mAb (B), or TRAIL (C), in the presence of different concentrations of dIGF-I. Cell death was measured by YO-PRO-1 fluorescence. RFU, relative fluorescence units. Bars, SEM; $n = 7$.

thus indicating the IGF-I-enhancing effect on cell death was due to IGF-IR activation and not to IR activation. Also note, that in the absence of death factor, dIGF-I did not induce changes in cell death by itself (not shown).

DNA fragmentation, a hallmark of apoptotic cells, was also assayed by flow cytometric analysis of propidium iodide-stained nuclei (Figure 2A) and quantification of nucleosomes released in the cytoplasm of cells (Figure 2B). These assays confirmed that dIGF-I can either inhibit or enhance cell apoptosis induced by TNF or anti-Fas and TRAIL, respectively. In addition, Figure 2C shows gene transcriptional profiles elicited by dIGF-I on treatment for 12 hours of IFN-sensitized cells with either TNF

Figure 2. IGF-I exerts both pro- and anti-apoptotic effects on TNFSF ligand-induced apoptosis. **A** and **B**: IFN-sensitized HT29-D4 cells were left either untreated (–) or incubated for 24 hours with TNF, anti-Fas mAb (Fas), or TRAIL in the absence (solid bars) or the presence of dIGF-I (open bars) at either 50 ng/ml (for TNF) or 10 ng/ml (for anti-Fas and TRAIL). Apoptosis was measured by flow cytometry for propidium iodide-stained nuclei (**A**), and determination by enzyme-linked immunosorbent assay of nucleosomal DNA fragmentation (**B**). $n = 3$. **C**: Variation of gene expression levels induced by dIGF-I in IFN-sensitized cells treated with either TNF or TRAIL for 12 hours, and quantified by cDNA expression array. Shown is the percentage of variation of the ratio of hybridization signals for (dIGF-I plus TNF-treated cells)/(TNF-treated cells) $\times 100$ (open bars) and (dIGF-I plus TRAIL-treated cells)/(TRAIL-treated cells) $\times 100$ (solid bars). Positive values indicate an up-regulation whereas negative values indicate a down-regulation of gene expression by dIGF-I. $n = 4$. $P < 0.05$ for all of the presented variations of gene expression. Bars, SEM.

or TRAIL. Twenty-seven genes were induced by either TNF or TRAIL but only 19 were significantly regulated by dIGF-I. As shown in Figure 2C, 17 of these dIGF-I-regulated genes were regulated for only one or for both TNFSF ligands, but with asymmetric effects. In particular, co-treatment of dIGF-I and TNF often resulted in down-regulation of proapoptotic genes, eg, Bax, Bak, caspase 9, and up-regulation of anti-apoptotic genes, eg, Rel-A, NF-κB1, GADD-45, whereas the opposite was observed for TRAIL. This mirror picture corroborates the above noted differential biological regulation of cell death by IGF-I depending on the TNFSF ligand used.

The Proapoptotic Activity of IGF-I Is Mediated by the PI3K/Akt Signaling Pathway

We previously reported^{14,15} that the IGF-I anti-apoptotic effect in IFN/TNF-stimulated HT29-D4 cells required a cooperation between Erk 1/2 MAPK, p38 MAPK, and NF-κB pathways in a manner independent of PI3K activity. To further study the signaling pathways involved in the proapoptotic effect of dIGF-I on apoptosis induced by anti-Fas and TRAIL, we used pharmacological inhibitors of several IGF-I-induced pathways: LY294002, a potent

Figure 3. IGF-I proapoptotic activity is conveyed via the PI3K pathway. **A** and **B**: HT29-D4 cells were preincubated without (–) or with LY294002 (LY), PD098059 (PD), SB203580 (SB), or Triptolide as indicated in Materials and Methods, then pulse-treated for 15 minutes with IFN, and further either left untreated (hatched bars) or treated for 24 hours with anti-Fas mAb (**A**) or TRAIL (**B**) in the absence (solid bars) or the presence (open bars) of 10 ng/ml dIGF-I. Cell death was measured by YO-PRO-1 fluorescence. RFU, relative fluorescence units. Bars, SEM; $n = 5$.

PI3K inhibitor; PD098059, a MAPK/Erk kinase 1 (MEK1) inhibitor; SB203580, a p38 MAPK inhibitor; and triptolide (PG490), an inhibitor of transcriptional activation of NF-κB. As a control, we noted that inhibition of PI3K, Erk 1/2, and p38 MAPK slightly decreased both anti-Fas- and TRAIL-induced apoptosis. In contrast, apoptosis was strongly enhanced by inhibiting NF-κB activation with triptolide (Figure 3, A and B).

As can be seen in Figure 3, LY294002 totally suppressed the enhancement of anti-Fas- and TRAIL-induced cell death by dIGF-I (Figure 3, A and B, respectively). In contrast, PD098059, SB203580, and triptolide had no effect on the dIGF-I-induced enhancement of cell death when compared to inhibitor-untreated cells. Note that quite similar results were observed whether cells were presensitized (Figure 3) or not (data not shown) with IFN. Thus, PI3K appears to be involved in IGF-I signaling pathways that enhance the levels of cell death induced by anti-Fas or TRAIL stimulation. In contrast, neither Erk 1/2 and p38 MAPK nor NF-κB participate to the proapoptotic action of IGF-I.

Figure 4. IGF-I enhances anti-Fas- and TRAIL-induced cell death in a PI3K-dependent manner in several human colon carcinoma cell lines. Cells were preincubated without (solid bars) or with (open bars) LY294002, then either left untreated (control) or exposed for 24 hours to anti-Fas mAb (A) or TRAIL (B) in the presence or in the absence of 10 ng/ml dIGF-I (treated cells). Cell death was measured by YO-PRO-1 fluorescence. Data in RFU (relative fluorescence units) were recorded as the difference in cell death (mean RFU) between treated and cognate control cells (Δ RFU). The percent modulation of cell death by dIGF-I was determined using the formula: $(\Delta$ RFU death factor plus dIGF-I-treated cells) - (Δ RFU death factor-treated cells) / (Δ RFU death factor-treated cells) $\times$ 100. Positive values indicate a proapoptotic effect whereas negative values indicate an anti-apoptotic effect of dIGF-I. *SW 620 cells do not express Fas.²⁵ **HRT-18 cells were presensitized by IFN. Bars, SEM; $n = 3$.

IGF-I Enhances Cell Death Response to Anti-Fas and TRAIL in Several Human Colon Cancer Cell Lines

Our results showing the existence of an unexpected PI3K-mediated proapoptotic effect of IGF-I in HT29-D4 cells raise the question of whether this effect is cell-specific or it occurs in other colon cancer cells. To examine these possibilities, we selected nine human colon cancer cell lines, which have been reported to express IGF-IR (unpublished results).^{14,19,24,25} As shown in Figure 4, dIGF-I enhanced anti-Fas-induced (Figure 4A) and TRAIL-induced (Figure 4B) cell death in five and six, respectively, of the nine cell lines analyzed (note that SW 620 cells do not express Fas²⁵). In SW 480 cells, Fas-induced apoptosis was not altered by dIGF-I whereas in HRT-18 rectum cancer cells, dIGF-I altered neither anti-

Fas- nor TRAIL-induced cell death (Figure 4). In addition, when detected, the proapoptotic effect of dIGF-I was blocked by LY294002 in all except in TRAIL-treated SW 480 cells (Figure 4). Finally, of all these cell lines, only COLO 205 cells acquired a significant resistance to apoptosis induced by either anti-Fas or TRAIL on dIGF-I addition. Moreover, this anti-apoptotic effect was not inhibited by LY294002, thus suggesting that it was not mediated by the PI3K pathway. Thus, although a relatively low number of tested colorectal cancer cell lines, these results suggest that a cell phenotype associated with up-regulation of TNFSF ligand-induced cell death by IGF-I may be encountered in a not uncommon manner in human colorectal tumors.

Evidence that Localization of IGF-IR in Lipid Rafts Controls Its Differential Signaling on Apoptosis

To explain the differential pro- versus anti-apoptotic signaling of IGF-I, we hypothesized that IGF-IR as well as IGF-IR-dependent signaling elements could be distributed in distinct membrane microdomains. It is indeed admitted that the plasma membrane is not homogenous and contains sphingolipid- and cholesterol-enriched discrete liquid-ordered (lo) microdomains, known as lipid rafts. Rafts have been shown to profoundly influence receptor-induced signal transduction and in turn the quality of biological responses.^{26,27} To test such a hypothesis, we first examined membrane partitioning of endogenous IGF-IR in lipid rafts.

Constitutive Partitioning of IGF-IR in Membrane Lipid Rafts

To examine the distribution of IGF-IR in lipid raft and nonraft membrane compartments, we used a procedure that extracts membrane rafts on the basis of their resistance to Brij 98 solubilization at 37°C.²⁰ Optiprep floatation gradients were then performed to isolate detergent-insoluble membranes that float to the low-density fractions while the solubilized material remains in the heavy fractions at the bottom of the gradient.

As shown in Figure 5A, the well-established raft-associated molecules, caveolin 1, GM1 glycosphingolipid, and Src family kinases, were enriched in the low-density fractions 2 to 3 of the gradient. In contrast, the non-raft protein clathrin heavy chain was totally solubilized and was always found to be associated with the heavier fractions 7 to 9, thus confirming the correct partitioning of different membrane proteins in the gradient (Figure 5A). Immunoblotting with anti-IGF-IR Ab showed that a significant part of IGF-IR was present in lipid raft fractions ($24.8 \pm 4.2\%$ as determined by densitometric analysis from six preparations). dIGF-I stimulation, in a range of 50 to 1000 ng/ml, did not induce any translocation of IGF-IR into or out of lipid rafts (not shown).

Acute disruption of lipid rafts can be accomplished through the extraction of cholesterol from membranes

Figure 5. Constitutive partitioning of IGF-IR in membrane rafts. HT29-D4 cells were left untreated (–) (A), cholesterol depleted with Me- β -CD (+) (B), or treated with Me- β -CD (+Chol.), then incubated with cholesterol/Me- β -CD complexes to replenish cholesterol (C), then postnuclear supernatant was treated with Brij 98 at 37°C and fractionated on an Optiprep floatation gradient (for more details, see Materials and Methods). Proteins from equal volumes from each fraction were analyzed by Western blotting using specific Abs against β -chain of IGF-IR, caveolin 1, clathrin heavy chain (H-clathrin), and panSrc. GM1 was detected using HRP-conjugated cholera toxin B chain. Shown is a representative experiment of six individual experiments.

of intact cells with Me- β -CD.^{26,28} Treatment of cells with Me- β -CD at 10 mmol/L for 30 minutes at 37°C reduced by ~50% the cholesterol content of plasma membrane (not shown). Although with this degree of cholesterol depletion, this treatment did not efficiently delocalize caveolin 1 from the light density fractions (Figure 5B). Such an insensitiveness has been previously reported, especially in epithelial cells.²⁸ In contrast, Me- β -CD treatment induced a drastic shift of GM1 and Src from the buoyant fractions to the high-density fractions in the gradient, thus confirming the disruption of some rafts in the cell membrane (Figure 5B). The same treatment also abolished the presence of IGF-IR in the lipid raft fraction and totally shifted it to the high-density fractions (Figure 5B) further confirming the association of an IGF-IR pool within a membrane raft subset. To ensure that this was a specific effect of cholesterol depletion, cells were first treated with Me- β -CD and subsequently repleted with cholesterol. As shown in Figure 5C, the shift in the localization of GM1, Src, and IGF-IR was fully reversed by cholesterol repletion with cholesterol/Me- β -CD complexes, indicating that association of these molecules, and especially IGF-IR, with detergent resistant membrane microdomains is actually cholesterol-dependent. Altogether, these data support the idea that two pools of IGF-IR are present in the plasma membrane of colon carcinoma cells, one constitutively resides in detergent-resistant, cholesterol-dependent microdomains that exist at physiological temperature, and another that is present out of these lipid rafts.

Membrane Lipid Rafts Control the Proapoptotic Effect of IGF-I

The compartmentalization of IGF-IR in distinct plasma membrane domains, one of them exhibiting the hallmarks of lipid rafts, prompted us to search for a cholesterol dependence of the IGF-I-mediated differential regulatory effect on apoptosis induced by different TNFSF ligands. Disruption of lipid rafts with Me- β -CD did not affect basal cell viability (Figure 6) or restore cell susceptibility to TNF-mediated cell killing in cells

Figure 6. Differential sensitivity to cholesterol depletion of pro- versus anti-apoptotic effect of IGF-I. HT29-D4 cells were incubated without (–) or with (+) Me- β -CD, or treated with Me- β -CD, then incubated with cholesterol/Me- β -CD complexes (+Chol.), then pulse-treated for 15 minutes with (+) or without (–) IFN. Cells were further left either untreated (hatched bars) or treated with TNF (A), anti-Fas mAb (B), and TRAIL (C), for 24 hours in the absence (solid bars) or the presence (open bars) of dIGF-I at either 50 ng/ml (for TNF) or 10 ng/ml (for anti-Fas and TRAIL). Cell death was measured by YO-PRO-1 fluorescence. RFU: relative fluorescence units. Bars, SEM; $n = 5$.

that were not presensitized by IFN (not shown). Me- β -CD inhibited apoptosis by $\sim 20\%$ whatever the TNF-FS ligand used and whether cells were presensitized or not by IFN (Figure 6). As shown in Figure 6A, there was no change in sensitivity of IFN-sensitized, TNF-stimulated cells to the survival effect of dIGF-I compared to control cells whether cells were treated with Me- β -CD without or with a subsequent cholesterol repletion. In strong contrast, the proapoptotic effect of dIGF-I on anti-Fas- and TRAIL-induced apoptosis was totally suppressed by Me- β -CD cell treatment whether cells were presensitized or not by IFN (Figure 6, B and C). As also shown in Figure 6, B and C, this inhibitory effect of Me- β -CD was totally reversed by cholesterol repletion. In addition to Me- β -CD, the cell cholesterol content was also reduced by cholesterol oxidase, which totally suppressed dIGF-I-enhancement of anti-Fas mAb- and TRAIL-induced cell death (not shown). In contrast, α CD, which does not promote cholesterol efflux from the membrane, did not affect the proapoptotic effect of dIGF-I (not shown). Taken together, these data show that the integrity of lipid rafts is required for IGF-I to enhance TRAIL- and anti-Fas-induced cell apoptosis. In contrast, this integrity is not necessary for the IGF-I anti-apoptotic effect on IFN/TNF-induced apoptosis.

Effects of Cholesterol Depletion on IGF-IR Function and Downstream Signaling Pathways

To evaluate the significance of the specific requirement for raft integrity in the induction of the IGF-IR-induced proapoptotic effect, we next investigated whether cholesterol depletion may affect IGF-IR signaling. As shown in Figure 7A, Me- β -CD treatment did not affect the tyrosine-specific IGF-IR autophosphorylation induced by dIGF-I. It should be noted that an identical conclusion can be drawn from experiments done with lysis buffer containing either 1% Triton X-100 or 0.3% deoxycholic acid plus 60 mmol/L ODG. ODG is a gentle nonionic detergent that is very efficient in solubilizing proteins associated with rafts.²⁶ Thus, we concluded that cholesterol depletion does not adversely affect IGF-IR activation nor its ability to autophosphorylate. We therefore next examined the ability of IGF-IR to tyrosine phosphorylate its immediate downstream substrate, IRS-1. Cholesterol depletion of cells resulted in a relative reduction in IRS-1 tyrosine-specific phosphorylation ($62.3 \pm 5.8\%$, $n = 4$) in response to dIGF-I treatment (Figure 7B). Thus, intact lipid rafts are required for at least a part of signal transfer from IGF-IR to IRS-1. As an expected consequence of the partial inhibition of IRS-1 phosphorylation, cholesterol depletion also significantly inhibited ($67.3 \pm 5.4\%$, $n = 6$) dIGF-I-induced phosphorylation/activation of Akt, the direct downstream target of PI3K (Figure 8A). This inhibition was fully reversible by replenishment of cellular cholesterol levels, which is consistent with reformation of raft microdomains (Figure 8A). On the other hand, Figure 8 shows that the same Me- β -CD treatment of cells did not

Figure 7. Cholesterol depletion inhibits IGF-I-induced tyrosine phosphorylation of IRS-1 but not tyrosine autophosphorylation of IGF-IR. HT29-D4 cells were incubated with (+) or without (–) Me- β -CD, then incubated for 2 minutes (**A**) or 10 minutes (**B**) with the indicated dose of dIGF-I. Cells were then lysed with 0.3% deoxycholic acid plus 60 mmol/L ODG, and lysates issued from an identical number of cells were immunoprecipitated (IP) with either anti-IGF-IR β chain Ab (**A**) or anti-IRS-1 (**B**), followed by immunoblotting with anti-phosphotyrosine Ab (WB: PY). The blots were then stripped and reprobed with anti-IGF-IR β chain Ab (WB: IGF-IR) (**A**) or anti-IRS-1 Ab (WB: IRS-1) (**B**). Shown is a representative experiment of four individual experiments.

inhibit IGF-I-induced phosphorylation/activation of p38 (Figure 8B) and Erk 1/2 (Figure 8C) MAPK.

Taken together, these data suggest that integrity of cholesterol-rich membrane rafts is necessary not for the activation of IGF-IR itself, but for a further full downstream signal propagation via IRS-1 phosphorylation and PI3K/Akt pathway activation, ie, the pathway we showed above to convey the IGF-I proapoptotic effect. They also show that, in contrast, disruption of lipid rafts did not affect the IGF-IR-induced signaling via Erk 1/2 and p38 MAPK, that we previously reported to convey the IGF-I anti-apoptotic effect.^{14,15}

Figure 8. Cholesterol depletion inhibits IGF-I-induced phosphorylation/activation of Akt, but not of p38 and Erk 1/2 MAPK. HT29-D4 cells were incubated without (–) or with (+) Me- β -CD or treated with Me- β -CD, then incubated with cholesterol/Me- β -CD complexes (+Chol.). Cells were then incubated for 15 minutes with the indicated dose of dIGF-I, then lysed with Triton X-100, and lysates issued from an identical number of cells were submitted to Western blot analysis to detect phosphorylated Akt (p-Akt) (**A**), phosphorylated p38 MAPK (p-p38) (**B**), and phosphorylated Erk 1/2 MAPK (p-Erk) (**C**). The blots were then stripped and reprobed with anti-Akt (Akt) (**A**), anti-p38 MAPK (p38) (**B**), and anti-Erk 1/2 MAPK (Erk) (**C**). Shown is a representative experiment of six (**A**) and three (**B**, **C**) individual experiments.

Figure 9. IGF-I stimulation induces IGF-IR autophosphorylation, then recruitment into and phosphorylation within lipid rafts of IRS-1 and Akt. HT29-D4 cells were incubated with (+) or without (–) Me-β-CD, then treated for the indicated time with dIGF-I, and then extracted with Brij 98 for 5 minutes at 37°C as described in Materials and Methods. Brij 98 soluble (S) and insoluble membrane raft (R) fractions were isolated and equal aliquots of the fractions were submitted to Western blot analysis to detect phosphorylated IGF-IR (p-IGF-IR), phosphorylated IRS-1 (p-IRS-1), phosphorylated Akt (p-Akt), and phosphorylated Erk 1/2 (p-Erk). The blots were then stripped and reprobed with anti-IGF-IR β-chain (IGF-IR), anti-IRS-1 (IRS-1), anti-Akt (Akt), anti-Erk 1/2 (Erk), and anti-caveolin 1. Shown is a representative experiment of two individual experiments.

IGF-I induced IGF-IR, IRS-1, and Akt but Not Erk Phosphorylation/Activation within Lipid Rafts

Having documented the partitioning of IGF-IR in lipid rafts and the cholesterol dependence of the IGF-I-induced PI3K/Akt-mediated proapoptotic effect, we next investigated if signaling molecules involved in this pathway were localized and activated within the raft fraction. In agreement with the constitutive membrane distribution of IGF-IR (Figure 5), autophosphorylation of IGF-IR was detected within lipid raft (R) and non-raft (S) fractions as soon as 2 minutes after dIGF-I stimulation (Figure 9, left). In unstimulated cells, IRS-1, Akt, and Erk were only present in non-raft fractions that contain detergent-soluble membrane proteins and cytosolic proteins. However, after 2 minutes of dIGF-I stimulation, we observed a significant recruitment of IRS-1 and Akt into rafts where they were phosphorylated with a maximum after 15 minutes of stimulation (Figure 9, left). In contrast, dIGF-I did not induce any recruitment of Erk into lipid rafts, and dIGF-I-induced Erk phosphorylation/activation was observed in non-raft fractions only (Figure 9, left). In agreement with the above reported effect of Me-β-CD on IGF-IR membrane partitioning (Figure 5), IGF-IR totally shifted into the non-raft compartment of the plasma membrane in Me-β-CD-treated cells (Figure 9, right). As a consequence, dIGF-I-induced recruitment of IRS-1 and Akt signaling molecules into rafts was completely abrogated as was impaired their phosphorylation/activation in this membrane compartment whereas non-raft Erk phosphorylation remained unaffected (Figure 9, right). Thus, IGF-IR within lipid rafts is functional and such a membrane location is necessary to initiate IRS-1 and Akt phosphorylation/activation within lipid rafts. However, a paral-

lel Me-β-CD-insensitive IGF-IR/IRS-1/Akt pathway was also activated in the non-raft fraction, which correlated with the constitutive presence of a large part of IGF-IR out of rafts (Figure 9). Collectively, these results indicate that a functional IGF-IR/IRS-1/Akt signaling pathway can be initiated by IGF-I in lipid rafts, thus strengthening our hypothesis that this pathway represents, at least in part, the molecular support for the proapoptotic signaling of IGF-I.

Discussion

The results presented in this article can be summarized as follows: 1) in colon carcinoma cells, the IGF-IR can induce either resistance to or enhancement of cell death depending on the TNFSF ligand used, here, TNF versus TRAIL and anti-Fas, respectively; 2) the paradoxical proapoptotic action of IGF-I is conveyed via the PI3K/Akt pathway; and 3) IGF-IR is constitutively located in and out of lipid rafts. Integrity of lipid rafts is however necessary for both the IGF-I proapoptotic effect and for the activation of the PI3K/Akt pathway in these microdomains. In contrast, the activation of Erk 1/2 and p38 MAPK pathways that convey the IGF-I-anti-apoptotic signaling^{14,15} occurs independently of lipid rafts.

Interestingly, we also report that the IGF-I proapoptotic effect on cell death triggered by anti-Fas or TRAIL is observed in five and six, respectively, of the nine tested human colon carcinoma cell lines. Although the relatively low number of tested cell lines does not allow to draw a general rule, it remains that such a proapoptotic effect of IGF-I could be not infrequent if one takes into account the large phenotypic heterogeneity of colon cancer cells within a tumor mass.

The Proapoptotic Effect of IGF-I

Initially, we hypothesized that IGF-I should act as an anti-apoptotic factor whatever the TNFSF death factor used to trigger cell death because of the numerous reported lines of evidence showing that the activated IGF-IR has a potent anti-apoptotic activity against a wide variety of stress stimuli in different systems of normal and cancer cells.^{6–9,12,13} However, an interesting finding of this study is that this rule is not absolute. Indeed, we showed in colon cancer cells that IGF-I could act as an anti-apoptotic factor when cell death was induced by TNF (the present work),^{14,15} but could also act as a proapoptotic factor when apoptosis was initiated by anti-Fas or TRAIL. It is worth noting that IGF-I did not induce apoptosis by itself; its proapoptotic function, measured here by three different assays, is to facilitate apoptosis triggered by either anti-Fas or TRAIL. Although unexpected, it is however not the first time that IGFs have been noted to act in a proapoptotic manner depending on the experimental conditions. Thus, IGF-I has been reported to increase TNF-induced apoptosis in skeletal myoblast and preadipocytes,^{29,30} Fas-induced apoptosis in human osteoblasts³¹ and caspase 3 activation, annexin-V binding, and DNA fragmentation in osteosar-

coma cells.³² Both IGF-I and insulin have also been shown to increase apoptosis in serum-starved glioma, hepatoma, and Wilms' tumor cells,^{33,34} and insulin has been reported to activate caspase 3 and induce apoptosis of myeloma cells.³⁵ Thus, the rule that growth factors protect cells from apoptosis is not without exceptions. This is true not only for the IGF system, but also for other growth factors such as the hepatocyte growth factor that was recently shown to sensitize human ovarian carcinoma cells to drug-induced apoptosis and to induce death of ovarian epithelial cells when extracellular matrix or intercellular contacts are lacking.³⁶

It is interesting to note that previous reports have given some molecular basis related to prodeath signals from the IGF-IR. Thus, expression of a membrane-targeted C-terminal construct of the IGF-IR induced massive cell death by a mechanism that is not a competitive inhibition of the IGF-IR but a proper proapoptotic signaling.^{37,38} Interestingly, to induce apoptosis, the isolated C-terminus of IGF-IR had to be myristylated, a well known lipid modification to specifically target signaling proteins to membrane rafts.²⁶ This finding thus suggests that the death-promoting signal arising from the IGF-IR may be due to its unique insertion in a cholesterol-rich membrane raft, which might allow a functional interaction of the C-terminus with the proapoptotic machinery of the cell (see discussion below). Although the molecular mechanism related to the prodeath signaling from the IGF-IR C-terminus still remains unclear, it has been shown that a 17-amino acid peptide derived from the C-terminus sequence of the IGF-IR was able to induce apoptosis in fibroblasts by activating the caspase cascade at the mitochondrial level.³⁹ This latter finding is also of interest given we observed that IGF-I added to TRAIL-stimulated HT29-D4 cells increased the expression of proapoptotic genes involved in the mitochondrial arm of the apoptotic pathway, eg, Bax, Bak, caspase 9, whereas their expression was down-regulated when IGF-I was added to TNF-stimulated cells. These results are also in agreement with a recent report showing a positive correlation between IGF-IR and proapoptotic Bax and Bak proteins in patients with colorectal cancer.⁴⁰

The PI3K/Akt Pathway Conveys the Proapoptotic Effect of IGF-I

Another somewhat unexpected result of this study is that activation of the PI3K/Akt pathway constitutes the molecular signaling support of the IGF-I-enhancing effect on anti-Fas- and TRAIL-induced cell death. The proapoptotic effect of IGF-I was indeed totally abrogated—except in SW 480 cells—by LY294002, a well established specific inhibitor of PI3K, the efficiency of which to totally inhibit the phosphorylation of Akt, its immediate downstream substrate, was routinely verified. The view that PI3K/Akt activation may promote cell death rather than survival during conditions of stress contradicts a number of other reports in which the PI3K/Akt pathway is critically involved in cell survival and prevention of apoptosis.⁴¹ However, several reports have also described instances

in which the activation of the PI3K/Akt pathway was not required for cell survival.^{42–44} We have also previously shown that the PI3K/Akt pathway activated by IGFs was not required for mediating the IGF-I anti-apoptotic function against IFN/TNF-induced apoptosis^{14,15} whereas it was involved in IGF-I-induced cell migration⁴⁵ in HT29-D4 colon carcinoma cells. In this study, we also observed that the survival effect of IGF-I on COLO 205 cells was not reversed by LY294002. This PI3K/Akt independence of the IGF-I anti-apoptotic function has also been observed in other cell models.^{46–48} Moreover, the novel view that PI3K/Akt activation promotes apoptosis rather than survival in certain conditions of stress has also been described. For example, the proapoptotic effect of IGF-I in preadipocytes stimulated by TNF requires activation of PI3K,²⁹ and the PI3K/Akt pathway is also capable to mediate insulin induction of apoptosis in myeloma cells.³⁵ In neutrophils, the transient activation of PI3K is also required for Fas-induced apoptosis⁴⁹ and IL-4 has been reported to facilitate the anisomycin-induced apoptosis of follicular dendritic cells by activating PI3K.⁵⁰ These controversial reported findings about PI3K function raise the possibility of conflicting roles of different classes and isoforms of PI3K and Akt in cancer cells. Thus, in colon carcinoma cells, PI3K α is involved in cell survival, PI3K β in DNA synthesis⁵¹ whereas PI3K γ can down-regulate expression of the anti-apoptotic molecule Bcl-2 and suppress the development of colorectal cancer *in vivo*.⁵² In a same way, inhibition of PI3K by wortmannin, which down-regulates NF- κ B, a well-known anti-apoptotic transcription factor in a lot of cell types, has been shown in contrast to increase NF- κ B binding activity in colon cancer cells.⁵³ Moreover, a cross-talk between PI3K/Akt activity and stress-regulated MAPK cascades has been shown in a number of cell systems. Often, Akt has been reported to inhibit activation of the kinases involved in these cascades.⁴¹ Thus, it could be argued that the proapoptotic property of PI3K/Akt signaling observed in colon cancer cells can be related to a proper equilibrium between activities of distinct classes of PI3K/Akt and other pathways involving Erk, p38 MAPK, or NF- κ B, that we reported to play a role in the IGF-I anti-apoptotic signal.^{14,15} However, the molecular mechanisms related to the PI3K/Akt prodeath signal as its detailed regulation in the cell still remain unclear and their understanding requires more investigation.

Lipid Rafts Segregate the Pro- and Anti-Apoptotic Signaling of IGF-I

Lipid rafts are discrete sphingolipid- and cholesterol-rich liquid-ordered microdomains of the plasma membrane that have been proposed to be efficient sites for the initiation of specific signal transduction due to the ability of activated receptors to access to particular downstream signaling components, while excluding them from others.^{26,27} For instance, lipid rafts have been recently reported to mediate Akt-regulated survival in prostate cancer cells both *in vitro* and in xenograft tumors.⁵⁴ Interestingly, in caveolin 1-overexpressing HeLa cells and

kidney fibroblasts, Akt can conversely trigger a death-promoting signal.⁵⁵ Caveolin 1 is the main structural component of caveolae, a subset of lipid membrane rafts.²⁶ Thus, we hypothesized that the distribution of IGF-IR in and out of lipid rafts may regulate the outcome of the IGF-IR-dependent signaling, and thus modulate in a differential manner the apoptotic response induced by TNFSF ligands in colon carcinoma cells.

To isolate lipid rafts, we used here a raft isolation procedure based on their resistance at 37°C to Brij 98 detergent extraction²⁰ allowing us to specifically analyze those rafts that are present at physiological temperature. Under these conditions, we found that ~25% of IGF-IR were constitutively localized in lipid rafts. On IGF-I stimulation, IGF-IR was rapidly activated whether it was located in or out of lipid rafts as indicated by the insensitivity of IGF-IR tyrosine autophosphorylation to cholesterol depletion. Thus, IGF-IR is capable of initiating signaling from both raft and non-raft regions of the plasma membrane, but we hypothesized that each compartment may direct a distinct signaling response.

Accordingly, we showed that disruption of lipid rafts with Me- β -CD inhibited the capacity of IGF-I to enhance apoptosis induced by anti-Fas or TRAIL and to induce a full activation of PI3K/Akt. These effects were likely the results of alterations in membrane cholesterol, because they were reversed by repletion of cell membrane with cholesterol. Thus, IGF-I proapoptotic signaling appears to be conveyed by a PI3K/Akt pathway that is under the control of IGF-IR located within lipid rafts. In agreement, we showed that on IGF-IR activation within rafts, the immediate downstream substrate IRS-1, and Akt were recruited to and phosphorylated/activated within these microdomains, and this process was totally impaired by disruption of rafts with Me- β -CD. Although raft-specific resident molecular targets and regulators of Akt are still unknown, it is interesting to note that a DNA-dependent protein kinase (DNA-PK) has been recently reported to function as an Akt-hydrophobic motif Ser-473 kinase in lipid rafts.⁵⁶ In contrast, lipid raft disruption did not affect the ability of IGF-I to inhibit TNF-induced apoptosis, a process we previously reported to be conveyed by Erk 1/2 and p38 MAPK, in a manner independent of PI3K.^{14,15} Accordingly, we show here that phosphorylation/activation of these MAPKs occurred under the control of IGF-IR out of rafts and, as expected, was not affected by cholesterol depletion.

Collectively, these findings suggest the involvement of an IGF-IR/IRS-1/PI3K/Akt signaling pathway that conveys the proapoptotic effect of IGF-I under the control of lipid rafts whereas the anti-apoptotic signaling of IGF-I mediated by Erk 1/2 and p38 MAPK pathways^{14,15} occurs independently of lipid rafts. A recent report showing that IGF-IR-induced PI3K/Akt and Raf/Erk pathways can transmit opposing signals on matrix metalloproteinase-2 expression⁵⁷ is in agreement with these findings. Moreover, membrane rafts and caveolae have been previously reported to be involved in IGF-IR and IR signaling in different cell systems.^{58–61} Some of these reports^{58,60} have also shown that these membrane microdomains are able to sort for PI3K/Akt versus MAPK signal transduction

pathways. Further studies are however necessary to analyze in detail the mechanisms of cross-talk between raft-dependent and -independent IGF-I signaling pathways and signals induced by the different TNFSF DR that have also been reported to depend on membrane microdomains.^{62,63}

Finally, our findings raise some intriguing questions about their putative clinical implications. First, they show that uncontrolled IGFs autocrine/paracrine loops established in the microenvironment of the cancer cell may enhance the deleterious role of TNF as a tumor promoter¹⁸ by inhibiting its proapoptotic activity and enhancing its proinflammatory and tumor-cell invasive effects.^{14,15} Conversely, they indicate that IGFs may also in some circumstances exert a tumor suppressor effect. Indeed, we show that IGF-I may increase apoptosis induced by FasL and TRAIL, ie, the best apoptosis-inducing weapons the immune system uses to eradicate cancer cells.^{3,4,16,17} Therefore, IGFs or IGF derivatives might be used in some circumstances to improve response to immunotherapy in colon carcinoma, which appears as a particularly intriguing possibility. Nonetheless, although the development of drugs inhibiting the activity of PI3K-Akt pathway⁶⁴ or the IGF-IR tyrosine kinase activity^{9,65} represents a potentially exciting new therapy, it should be remembered that both IGF-IR and PI3K/Akt may perform functions that we might not want to inhibit and thus appropriate caution should be taken.

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References

1. Danial NN, Korsmeyer SJ: Cell death: critical control points. *Cell* 2004, 116:205–219
2. Thompson CB: Apoptosis in the pathogenesis and treatment of disease. *Science* 1995, 267:1456–1462
3. Igney FH, Krammer PH: Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer* 2002, 2:277–288
4. Reed JC: Apoptosis-targeted therapies for cancer. *Cancer Cell* 2003, 3:17–22
5. Hanahan D, Weinberg RA: Cell. The hallmarks of cancer. *Cell* 2000, 100:57–70
6. Baserga R, Peruzzi F, Reiss K: The IGF-1 receptor in cancer biology. *Int J Cancer* 2003, 107:873–877
7. Werner H, Le Roith D: New concepts in regulation and function of the insulin-like growth factors: implications for understanding normal growth and neoplasia. *Cell Mol Life Sci* 2000, 57:932–942
8. Zhang D, Samani AA, Brodt P: The role of the IGF-I receptor in the regulation of matrix metalloproteinases, tumor invasion and metastasis. *Horm Metab Res* 2003, 35:802–808
9. Surmacz E: Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor I receptor. *Oncogene* 2003, 22:6589–6597
10. Pollak MN, Schernhammer ES, Hankinson SE: Insulin-like growth factors and neoplasia. *Nat Rev Cancer* 2004, 4:505–518
11. Jones JL, Clemmons DR: Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev* 1995, 16:3–34
12. Baserga R: The contradictions of the insulin-like growth factor 1 receptor. *Oncogene* 2000, 19:5574–5581

13. Blakesley VA, Butler AA, Koval AP, Okubo Y, LeRoith D: IGF-I receptor function: transducing the IGF-I signal into intracellular events. The IGF System. Edited by Rosenfeld RG, Roberts CT. Totowa, Humana Press, 1999, pp 143–163
14. Remacle-Bonnet MM, Garrouste FL, Heller S, Andre F, Marvaldi JL, Pommier GJ: Insulin-like growth factor-I protects colon cancer cells from death factor-induced apoptosis by potentiating tumor necrosis factor alpha-induced mitogen-activated protein kinase and nuclear factor kappaB signaling pathways. *Cancer Res* 2000, 60:2007–2017
15. Garrouste F, Remacle-Bonnet M, Fauriat C, Marvaldi J, Luis J, Pommier G: Prevention of cytokine-induced apoptosis by insulin-like growth factor-I is independent of cell adhesion molecules in HT29–D4 colon carcinoma cells—evidence for a NF-kappaB-dependent survival mechanism. *Cell Death Differ* 2002, 9:768–779
16. Aggarwal BB: Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 2003, 3:745–756
17. Ashkenazi A: Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2002, 2:420–430
18. Szlosarek PW, Balkwill FR: Tumour necrosis factor alpha: a potential target for the therapy of solid tumours. *Lancet Oncol* 2003, 4:565–573
19. Andre F, Janssens B, Bruyneel E, van Roy F, Gespach C, Mareel M, Bracke M: Alpha-catenin is required for IGF-I-induced cellular migration but not invasion in human colonic cancer cells. *Oncogene* 2004, 23:1177–1186
20. Drevot P, Langlet C, Guo XJ, Bernard AM, Colard O, Chauvin JP, Lasserre R, He HT: TCR signal initiation machinery is pre-assembled and activated in a subset of membrane rafts. *EMBO J* 2002, 21:1899–1908
21. Shigematsu S, Watson RT, Khan AH, Pessin JE: The adipocyte plasma membrane caveolin functional/structural organization is necessary for the efficient endocytosis of GLUT4. *J Biol Chem* 2003, 278:10683–10690
22. Klein U, Gimpl G, Fahrenholz F: Expression of the human oxytocin receptor in baculovirus-infected insect cells: high-affinity binding is induced by a cholesterol-cyclodextrin complex. *Biochemistry* 1995, 34:13794–13801
23. Garrouste FL, Remacle-Bonnet MM, Lehmann MM, Marvaldi JL, Pommier GJ: Up-regulation of insulin/insulin-like growth factor-I hybrid receptors during differentiation of HT29–D4 human colonic carcinoma cells. *Endocrinology* 1997, 138:2021–2032
24. Lahm H, Amstad P, Wyniger J, Yilmaz A, Fischer JR, Schreyer M, Givel JC: Blockade of the insulin-like growth-factor-I receptor inhibits growth of human colorectal cancer cells: evidence of a functional IGF-II-mediated autocrine loop. *Int J Cancer* 1994, 58:452–459
25. Suzuki A, Kusakai G, Kishimoto A, Shimojo Y, Miyamoto S, Ogura T, Ochiai A, Esumi H: Regulation of caspase-6 and FLIP by the AMPK family member ARK5. *Oncogene* 2004, 23:7067–7075
26. Simons K, Toomre D: Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* 2000, 1:31–39
27. Munro S: Lipid rafts: elusive or illusive? *Cell* 2003, 115:377–388
28. Schuck S, Honsho M, Ekroos K, Shevchenko A, Simons K: Resistance of cell membranes to different detergents. *Proc Natl Acad Sci USA* 2003, 100:5795–5800
29. Niesler CU, Urso B, Prins JB, Siddle K: IGF-I inhibits apoptosis induced by serum withdrawal, but potentiates TNF-alpha-induced apoptosis, in 3T3–L1 preadipocytes. *J Endocrinol* 2000, 167:165–174
30. Foulstone EJ, Meadows KA, Holly JM, Stewart CE: Insulin-like growth factors (IGF-I and IGF-II) inhibit C2 skeletal myoblast differentiation and enhance TNF alpha-induced apoptosis. *J Cell Physiol* 2001, 189:207–215
31. Kawakami A, Nakashima T, Tsuboi M, Urayama S, Matsuoka N, Ida H, Kawabe Y, Sakai H, Migita K, Aoyagi T, Nakashima M, Maeda K, Eguchi K: Insulin-like growth factor I stimulates proliferation and Fas-mediated apoptosis of human osteoblasts. *Biochem Biophys Res Commun* 1998, 247:46–51
32. Raile K, Hille R, Laue S, Schulz A, Pfeifer G, Horn F, Kiess W: Insulin-like growth factor I (IGF-I) stimulates proliferation but also increases caspase-3 activity, annexin-V binding, and DNA-fragmentation in human MG63 osteosarcoma cells: co-activation of pro- and anti-apoptotic pathways by IGF-I. *Horm Metab Res* 2003, 35:786–793
33. Yang BC, Chang HM, Wang YS, Chen RF, Lin SJ: Transient induction of apoptosis in serum-starved glioma cells by insulin and IGF-1. *Biochim Biophys Acta* 1996, 1314:83–92
34. Granerus M, Johannisson A, Ekblom P, Engstrom W: Insulin-like growth factors I and II induce cell death in Wilms's tumour cells. *Mol Pathol* 2001, 54:30–35
35. Godbout JP, Cengel KA, Cheng SL, Minshall C, Kelley KW, Freund GG: Insulin activates caspase-3 by a phosphatidylinositol 3'-kinase-dependent pathway. *Cell Signal* 1999, 11:15–23
36. Rasola A, Anguissola S, Ferrero N, Gramaglia D, Maffe A, Maggiora P, Comoglio PM, Di Renzo MF: Hepatocyte growth factor sensitizes human ovarian carcinoma cell lines to paclitaxel and cisplatin. *Cancer Res* 2004, 64:1744–1750
37. Liu Y, Lehar S, Corvi C, Payne G, O'Connor R: Expression of the insulin-like growth factor I receptor C terminus as a myristylated protein leads to induction of apoptosis in tumor cells. *Cancer Res* 1998, 58:570–576
38. Hongo A, Yumet G, Resnicoff M, Romano G, O'Connor R, Baserga R: Inhibition of tumorigenesis and induction of apoptosis in human tumor cells by the stable expression of a myristylated COOH terminus of the insulin-like growth factor I receptor. *Cancer Res* 1998, 58:2477–2484
39. Reiss K, Yumet G, Shan S, Huang Z, Alnemri E, Srinivasula SM, Wang JY, Morriore A, Baserga R: Synthetic peptide sequence from the C-terminus of the insulin-like growth factor-I receptor that induces apoptosis and inhibition of tumor growth. *J Cell Physiol* 1999, 181:124–135
40. Koda M, Reszec J, Sulkowska M, Kanczuga-Koda L, Sulkowski S: Expression of the insulin-like growth factor-I receptor and proapoptotic bax and bak proteins in human colorectal cancer. *Ann NY Acad Sci* 2004, 1030:377–383
41. Franke TF, Hornik CP, Segev L, Shostak GA, Sugimoto C: PI3K/Akt and apoptosis: size matters. *Oncogene* 2003, 22:8983–8998
42. Burns TF, El-Deiry WS: Identification of inhibitors of TRAIL-induced death (ITIDs) in the TRAIL-sensitive colon carcinoma cell line SW480 using a genetic approach. *J Biol Chem* 2001, 276:37879–37886
43. Galvan V, Logvinova A, Sperandio S, Ichijo H, Bredesen DE: Type 1 insulin-like growth factor receptor (IGF-IR) signaling inhibits apoptosis signal-regulating kinase 1 (ASK1). *J Biol Chem* 2003, 278:13325–13332
44. Bartucci M, Morelli C, Mauro L, Ando' S, Surmacz E: Differential insulin-like growth factor I receptor signaling and function in estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. *Cancer Res* 2001, 61:6747–6754
45. Andre F, Rigot V, Remacle-Bonnet M, Luis J, Pommier G, Marvaldi J: Protein kinases C-gamma and -delta are involved in insulin-like growth factor I-induced migration of colonic epithelial cells. *Gastroenterology* 1999, 116:64–77
46. Kulik G, Weber MJ: Akt-dependent and -independent survival signaling pathways utilized by insulin-like growth factor I. *Mol Cell Biol* 1998, 18:6711–6718
47. Thimmaiah KN, Easton J, Huang S, Veverka KA, Germain GS, Harwood FC, Houghton PJ: Insulin-like growth factor I-mediated protection from rapamycin-induced apoptosis is independent of Ras-Erk1-Erk2 and phosphatidylinositol 3'-kinase-Akt signaling pathways. *Cancer Res* 2003, 63:364–374
48. Leahy M, Lyons A, Krause D, O'Connor R: Impaired Shc, Ras, and MAPK activation but normal Akt activation in FL5.12 cells expressing an insulin-like growth factor I receptor mutated at tyrosines 1250 and 1251. *J Biol Chem* 2004, 279:18306–18313
49. Alvarado-Kristensson M, Porn-Ares MI, Grethe S, Smith D, Zheng L, Andersson T: p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase activities have opposite effects on human neutrophil apoptosis. *FASEB J* 2002, 16:129–131
50. Lee HM, Jin HS, Park JW, Park SM, Jeon HK, Lee TH: IL-4 augments anisomycin-induced p38 activation via Akt pathway in a follicular dendritic cell (FDC)-like line. *FEBS Lett* 2003, 549:110–114
51. Benistant C, Chapuis H, Roche SA: A specific function for phosphatidylinositol 3-kinase alpha (p85alpha-p110alpha) in cell survival and for phosphatidylinositol 3-kinase beta (p85alpha-p110beta) in de novo DNA synthesis of human colon carcinoma cells. *Oncogene* 2000, 19:5083–5090
52. Sasaki T, Irie-Sasaki J, Horie Y, Bachmaier K, Fata JE, Li M, Suzuki A, Bouchard D, Ho A, Redston M, Gallinger S, Khokha R, Mak TW, Hawkins PT, Stephens L, Scherer SW, Tsao M, Penninger JM: Colorectal carcinomas in mice lacking the catalytic subunit of PI(3)K. *Nature* 2000, 406:897–902
53. Wang Q, Kim S, Wang X, Evers BM: Activation of NF-kB binding in

- HT-29 colon cancer cells by inhibition of phosphatidylinositol 3-kinase. *Biochem Biophys Res Commun* 2000, 273:853–858
54. Zhuang L, Kim J, Adam RM, Solomon KR, Freeman MR: Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts. *J Clin Invest* 2005, 115:959–968
55. Shack S, Wang XT, Kokkonen GC, Gorospe M, Longo DL, Holbrook NJ: Caveolin-induced activation of the phosphatidylinositol 3-kinase/Akt pathway increases arsenite cytotoxicity. *Mol Cell Biol* 2003, 23:2407–2414
56. Hill MM, Feng J, Hemmings BA: Identification of a plasma membrane Raft-associated PKB Ser473 kinase activity that is distinct from ILK and PDK1. *Curr Biol* 2002, 12:1251–1255
57. Zhang D, Bar-Eli M, Meloche S, Brodt P: Dual regulation of MMP-2 expression by the type 1 insulin-like growth factor receptor: the phosphatidylinositol 3-kinase/Akt and Raf/ERK pathways transmit opposing signals. *J Biol Chem* 2004, 279:19683–19690
58. Podar K, Tai YT, Cole CE, Hideshima T, Sattler M, Hamblin A, Mitsiades N, Schlossman RL, Davies FE, Morgan GJ, Munshi NC, Chauhan D, Anderson KC: Essential role of caveolae in interleukin-6- and insulin-like growth factor I-triggered Akt-1-mediated survival of multiple myeloma cells. *J Biol Chem* 2003, 278:5794–5801
59. Huo H, Guo X, Hong S, Jiang M, Liu X, Liao K: Lipid rafts/caveolae are essential for insulin-like growth factor-1 receptor signaling during 3T3–L1 preadipocyte differentiation induction. *J Biol Chem* 2003, 278:11561–11569
60. Parpal S, Karlsson M, Thorn H, Stralfors P: Cholesterol depletion disrupts caveolae and insulin receptor signaling for metabolic control via insulin receptor substrate-1, but not for mitogen-activated protein kinase control. *J Biol Chem* 2001, 276:9670–9678
61. Saltiel AR, Pessin JE: Insulin signaling pathways in time and space. *Trends Cell Biol* 2002, 12:65–71
62. Hueber AO: Role of membrane microdomain rafts in TNFR-mediated signal transduction. *Cell Death Differ* 2003, 10:7–9
63. Muppidi JR, Tschopp J, Siegel RM: Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. *Immunity* 2004, 21:461–465
64. Vivanco I, Sawyers CL: The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002, 2:489–501
65. Garcia-Echeverria C, Pearson MA, Marti A, Meyer T, Mestan J, Zimmermann J, Gao J, Brueggen J, Capraro HG, Cozens R, Evans DB, Fabbro D, Furet P, Porta DG, Liebetanz J, Martiny-Baron G, Ruetz S, Hofmann F: In vivo antitumor activity of NVP-AEW541—a novel, potent, and selective inhibitor of the IGF-IR kinase. *Cancer Cell* 2004, 5:231–239