Ectopic expression of Wnt-1 in 3T3-L1 preadipocytes stabilizes β-catenin, activates TCF-dependent gene transcription, and blocks adipogenesis. Here we report that upon serum withdrawal, Wnt-1 causes 3T3-L1 cells to resist apoptosis through a mechanism that is partially dependent on phosphatidylinositol 3-kinase. Although activation of Wnt signaling by inhibition of GSK-3 activity or ectopic expression of dominant stable β-catenin blocks apoptosis, inhibition of Wnt signaling through expression of dominant negative TCF-4 increases apoptosis. Wnt-1 stimulates 3T3-L1 preadipocytes to secrete factors that increase PKB/Akt phosphorylation at levels comparable with treatment with 10% serum. With DNA microarrays, we identified several secreted antiapoptotic genes that are induced by Wnt-1, notably insulin-like growth factor I (IGF-I) and IGF-II. Consistent with IGFs mediating the antiapoptotic effects of Wnt-1 in preadipocytes, conditioned medium from Wnt-1 expressing 3T3-L1 cells was unable to promote protein kinase B phosphorylation after the addition of recombinant IGF-FBP-4. Thus, we demonstrated that Wnt-1 induces expression of antiapoptotic genes in 3T3-L1 preadipocytes such as IGF-I and IGF-II, which allows these cells to resist apoptosis in response to serum deprivation.

The Wnts are a family of proteins that affect cell fate and differentiation, including adipogenesis, myogenesis, neurogenesis, and mammary development (1–4). The Wnt-1 gene was first identified as an insertion site for mouse mammary tumor virus in mouse mammary carcinoma (5). Wnts are secreted glycoproteins that interact with seven transmembrane frizzled receptors and low density lipoprotein receptor-related protein co-receptors (6, 7). In the canonical Wnt signaling pathway, inhibition of GSK-3 prevents phosphorylation and targeted degradation of β-catenin. In the absence of Wnt signaling, hypophosphorylated β-catenin accumulates in the cytoplasm, enters the nucleus, and activates TCF/LEF-dependent gene transcription (8).

We previously reported that both Wnt-1 and Wnt-10b block adipogenic conversion of 3T3-L1 preadipocytes through stabilization of β-catenin and inhibition of C/EBPαs and peroxisome proliferator-activated receptor γ expression (9). Inhibition of Wnt signaling with dominant negative TCF-4 or with soluble frizzled-related proteins (sFRP) causes spontaneous differentiation (3, 9), indicating that an endogenous Wnt feeds back to repress adipogenesis. Wnt-10b is the best candidate for the endogenous inhibitor because Wnt-10b stabilizes free cytosolic β-catenin, inhibits adipogenesis, and is expressed in preadipocytes and stromovascular cells but not in adipocytes (3, 9).

Suppression of Wnt-10b in response to elevated cAMP promotes expression of adipogenic transcription factors and proteins involved in carbohydrate and lipid metabolism (3, 9–11).

In addition to playing a key role in adipogenesis, Wnt signaling protects against apoptosis in cells exposed to cellular or chemical stress (12–14). For example, ectopic expression of Wnt-1 in Rat-1 cells inhibits apoptosis in response to vincristine or vinblastine through a PKB/Akt-independent mechanism (13). Furthermore, low serum conditions fail to induce apoptosis in PC-12 cells that express Wnt-1 (12). Inhibitors of GSK-3 and PI3K each partially reversed this effect, suggesting that the cytoprotective effects of Wnt-1 are mediated through direct Wnt signaling and Wnt-induced gene expression (12).

The response of 3T3-L1 cells to cellular stress is differentiation-dependent. Serum-starved 3T3-L1 preadipocytes undergo apoptosis, but fully differentiated adipocytes are resistant, perhaps because of increased expression of Bcl-2 and neuronal apoptosis inhibitory protein (15). Supplementation of serum-free medium with IGF-I protects preadipocytes against apoptosis, indicating that this growth factor impacts this process (16). Wnt signaling has pleiotropic effects during development, including the regulation of cell fate and mitosis. Recently, it has been shown that this signaling pathway has an important antiapoptotic function. However, the molecular mechanism for the protective effects of Wnt have only been partially characterized. Herein, we demonstrate that Wnt-1 induces expression of antiapoptotic genes in 3T3-L1 preadipocytes such as IGF-I and IGF-II, which allows these cells to resist apoptosis in response to serum deprivation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Retroviral Gene Expression—**The 3T3-L1 preadipocyte line was cultured in Dulbecco's modified Eagle's medium supplemented with 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% calf serum (Sigma) (17). Cells were maintained in a subconfluent state unless otherwise indicated. Human hemagglutinin-tagged Wnt-1 in the retroviral vector pLNCX, compliments of Jan Kitajewski (Columbia University), was used to generate retroviral stocks in 293T cells for subsequent infection of 3T3-L1 cells. Pooled
clones were selected with 400 μg/ml Genetricin (Invitrogen Life Technologies). Dominant negative TCF-4 (ΔN31 TCF-4) and dominant stable β-catenin (333Y β-cat), each in the neomycin-resistant vector, pPGS, were provided by Eric Fearon (University of Michigan). Mouse recombinant IGF-I and IGF-II and human recombinant IGFBP-4 were purchased from R&D Systems. Wortmannin (Calbiochem) and LY294002 (New England Biolabs) were used to inhibit PI3K.

Detection of Apoptosis—Cells were analyzed for apoptosis by TUNEL. Control and Wnt-1 cells incubated in the presence of Wnt-1 or Wnt-1 retrovirus (Wnt-1). After selection in G418, pooled clones were cultured in growth medium until confluent and then cultured for 24 h in serum-free media (+ Serum) or media containing 10% calf serum (+ Serum). Apoptotic cells were visualized by TUNEL staining and fluorescent microscopy (A), and the apoptotic index and standard deviation for each treatment were determined (B). Treatment groups with connected bars differ significantly by Student’s t test (p < 0.01).

DNA Microarray Analysis—The use of DNA microarrays to analyze gene expression in 3T3-L1 cells has been described elsewhere (11). Total RNA was isolated with RNA Stat60 (Tel-Test “B,” Inc.) from 3T3-L1 cells infected with either pLNCX or pLNCX-Wnt-1. RNA was further purified with RNeasy spin columns (Qiagen). Preparation of cRNA and hybridization and scanning of the mouse genome U74A arrays were performed according to the manufacturer’s protocol (Affymetrix). Arrays were scanned at 3 mm using the GeneArray scanner (Affymetrix). Expression levels for ~10,000 genes were given as arbitrary units, and the unit ratio of Wnt-1 to pLNCX was regarded as the Wnt-1-mediated fold induction for each gene.

Analysis of IGF-I and IGF-II Gene Expression—The use of DNA microarrays to analyze gene expression in 3T3-L1 cells has been described elsewhere (11). Total RNA was isolated from confluent 3T3-L1 control cells (infected with pLNCX) or Wnt-1 expressing cells (infected with pLNCX-Wnt-1), and reverse transcribed with Superscript II (Invitrogen). PCR amplification was performed for IGF-1 (forward primer, 5′-aaattgacaagctgtaastanaaga-3′; reverse primer, 5′-aectggcttgtagtgcttcttc-3′), IGF-II (forward primer, 5′-tccggaasgggattagcatcagcttca-3′; reverse primer, 5′-agttggagcacttggattctg-3′; and GAPDH (forward primer, 5′-gactgctgggttgcg-3′; reverse primer, 5′-gctggttaacctggattgtg-3′) using AmpliTaq (Applied Biosystems, Inc.) Conditions for PCR were: an initial denaturation step (96°C, 4 min) followed by 30 amplification steps (96°C, 1 min; 55°C, 1 min; 72°C, 2 min) and a final elongation (72°C, 10 min). Cloned PCR products were sequenced to confirm the specificity of these primers. PCR products were separated by 0.7% agarose gel electrophoresis and visualized with ethidium bromide and UV excitation.

Western Blot Analysis—Cells were washed with ice cold 1× phosphate-buffered saline and solubilized in the following lysis buffer: 250 mM Tris-Cl, pH 6.8, 0.1% SDS, 20% glycerol, 10 mM EDTA, 2% 2-mercaptoethanol, 0.01% bromphenol blue, and a mixture of protease inhibitors (aprotinin, phenylmethylsulfonyl fluoride, leupeptin, chymostatin, and pepstatin-A (Sigma)). Equal amounts of total protein were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Immunoblots were performed with antibodies specific for Akt, phospho-Akt (Ser-473), and phospho-Forkhead (Ser-256) according to manufacturer’s instructions (Cell Signaling Technology) and developed using enhanced chemiluminescence (Pierce).

RESULTS

**Wnt-1 Prevents Apoptosis in 3T3-L1 Cells**—Several cell types that normally undergo apoptosis in response to cellular stress are protected by ectopic expression of Wnt-1 (12–14). To determine whether Wnt-1 signaling protects 3T3-L1 preadipocytes from apoptosis, we isolated control Wnt-1 or empty vector (pLNCX) were cultured in serum or serum-free media for 24 h, and subsequently evaluated for apoptosis with TUNEL. Control and Wnt-1 cells incubated in the presence of serum showed very low rates of apoptosis (<1%; Fig. 1). Although control cells incubated in the absence of serum had a high rate of apoptosis (~20%), apoptosis in response to serum deprivation was largely blocked by Wnt-1 (~3.5%). Thus, Wnt-1 signaling inhibits apoptosis in 3T3-L1 preadipocytes under serum-free conditions.

**Lithium, an Inhibitor of GSK-3, Blocks Apoptosis in 3T3-L1 Cells**—Although Wnt proteins can signal through numerous pathways (6), we assessed whether the canonical Wnt signaling pathway mediates the antiapoptotic response. Wnt-1 signaling negatively regulates the activity of the enzyme GSK-3. To test whether the antiapoptotic effect of Wnt-1 is mediated through inhibition of GSK-3, cells were serum-deprived in the presence of the GSK-3 inhibitor lithium. Inhibition of GSK-3 caused a profound reduction in the level of apoptosis in serum-deprived cells compared with controls (Fig. 2), suggesting that Wnt-1 inhibits apoptosis through activation of the canonical Wnt signaling pathway.

**Dominant Stable β-Catenin Is Antiapoptotic, whereas dnTCF-4 Is Proapoptotic**—Given that GSK-3 mediates the effects of Wnt-1 on apoptosis, we investigated whether proteins downstream of GSK-3, such as β-catenin and TCF-4, were also involved. We hypothesized that a stable mutant of β-catenin, which cannot be phosphorylated and degraded by the proteosomal complex, would block apoptosis. Conversely, we hypothesized that a dominant negative form of TCF-4, capable of interacting with β-catenin but incapable of binding DNA, would promote apoptosis. To test these hypotheses, we ectopically expressed, in 3T3-L1 cells, a dominant stable form of...
act through an autocrine/paracrine mechanism to activate the apoptosis by inducing expression and secretion of IGFs, which (Table I, Fig. 4). Thus, we hypothesized that Wnt-1 inhibits (16, 19), and Wnt-1 induces expression of IGF-I and IGF-II (dnTCF-4, and appropriate controls (pLNCX or pPGS) were exposed to serum-free medium for 24 h. Apoptotic cells were visualized by TUNEL staining and fluorescent microscopy, and the apoptotic index and standard deviation for each treatment were calculated. Treatment groups with connected bars differ significantly by Student’s t test (p < 0.01).

Ectopic Expression of Wnt-1 in 3T3-L1 Cells Induces Expression of Antiapoptotic mRNAs—To test the hypothesis that Wnt-1 promotes the expression of genes that are themselves antiapoptotic, we performed microarray analyses on RNA from 3T3-L1 cells infected with control retrovirus or a retrovirus that ectopically express Wnt-1 (pLNCX) was isolated from three independently derived clones of 3T3-L1 cells (38, 39). Reverse transcription PCR was performed for IGF-I, IGF-II, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or its control (pLNCX). GAPDH primers were designed to amplify a 141-bp fragment. PCR products were separated by 0.7% agarose gel electrophoresis in 1× Tris acetate/EDTA and visualized by ethidium bromide staining.

The ability of condition media from Wnt-1 cells strongly stimulated phosphorylation of PKB/Akt (Fig. 3). Phosphorylation of PKB/Akt and its downstream target, Forkhead (Fig. 5A). Phosphorylation of Forkhead inhibits the activity of this proapoptotic transcription factor (20). The ability of conditioned media from antiapoptotic PKB/Akt pathway. Conditioned media from Wnt-1 cells strongly stimulated phosphorylation of PKB/Akt and its downstream target, Forkhead (Fig. 5A). Phosphorylation of Forkhead inhibits the activity of this proapoptotic transcription factor (20).

We demonstrate that ectopic expression of Wnt-1 and activation of the canonical Wnt signaling pathway promotes the survival of 3T3-L1 cells during serum deprivation. Our experimental approach to elucidate the mechanism of this antiapoptotic response was to 1) ectopically express different components of the Wnt-1 signaling pathway in order to recapitulate or block the effects of Wnt-1 signaling on apoptosis, 2) perform DNA microarray analyses to establish whether the expression of antiapoptotic genes is increased by Wnt-1 signaling, and 3) assess the ability of target genes to activate antiapoptotic pathways in 3T3-L1 cells. We found that activation of Wnt signaling by inhibition of GSK-3 or expression of a gain-of-function mutant of β-catenin inhibited apoptosis. Conversely, inhibition of Wnt signaling with a dominant negative TCF-4 potentiated apoptosis. These results highlight the importance of the canonical Wnt signaling pathway in the regulation of preadipocyte apoptosis. Microarray analysis identified several candidate genes induced by Wnt-1 and that have known effects on antiapoptosis. Two of these genes, IGF-I and IGF-II, were characterized further. The secretion of IGFs by 3T3-L1 cells that ectopically express Wnt-1, and the ability of IGFs to activate the PKB/Akt pathway was confirmed using the IGF-neutralizing protein, IGFBP-4. Finally, a role for the IGF-I signaling pathway in the ability of Wnt-1 to inhibit apoptosis was established using PI3K inhibitors.

The ability of Wnt-1 to inhibit apoptosis has been explored in several cell types (12, 13). Ectopic expression of either dominant negative TCF-4, or a gain-of-function mutant of β-catenin, has profound effects on the survival of 3T3-L1 cells (Fig. 3) and Rat-1 cells (13) consistent with their roles as downstream effectors of Wnt-1. Another member of the TCF family of transcription factors, LEF-1, is involved in antiapoptosis and Wnt-mediated proliferation in B-lymphocytes. In LEF-1 nullizygous mice, B-lymphocytes have increased apoptosis, perhaps as a result of increased Fas and c-Myc expression (22). Treatment with lithium increased proliferation of wild type B-lymphocytes, whereas Wnt-3a was unable to induce cell proliferation in B-lymphocytes from LEF-1 nullizygous animals (22). Collectively, these data define a universal mechanism by which Wnt-mediated gene expression influences both antiapoptosis and proliferation in a variety of cell types.

Through DNA microarray analysis, we have identified several candidate genes that are induced by Wnt-1 and are antiapoptotic (Table I). We focused our efforts on two of these genes, IGF-I and IGF-II. In particular, IGF-I acts through a phosphoinositide-dependent protein kinase-1 (PDK1), and ultimately PKB/Akt (21). Active PKB/Akt phosphorylates several target proteins whose activation (e.g. IκB kinase) or inactivation (e.g. Bcl-2-antagonist of cell death, Forkhead, caspase 9) promotes cell survival (21). Given the potent IGF activity in conditioned media of 3T3-L1 cells that ectopically express Wnt-1, we hypothesized that the survival of these cells during serum starvation might be PI3K-dependent. Consistent with this hypothesis, 3T3-L1 cells that ectopically express Wnt-1 have elevated levels of apoptosis in the presence of the PI3K inhibitors wortmannin or LY294002 (Fig. 6). However, the intermediate levels of apoptosis in the PI3K inhibitor-treated cells suggests that other mechanisms, perhaps direct activation of antiapoptotic signaling pathways by Wnt-1, may also be involved.
Expression of Wnt-1 in mammary epithelial cells was associated with tumor promotion through several mechanisms, including inhibition of apoptosis (26). The regulation of COX-2 expression by Wnt-1 signaling was first described in Wnt-1-transformed mouse mammary epithelial cells (27). Expression of Wnt-1 in mammary epithelial cells was associated with stabilization of β-catenin, increased transcription of COX-2 mRNA, and increased synthesis of PGE2 (27). The influence of Wnt-1 on COX-2 expression is likely to be mediated by the transcription factor PEA3, which is positively regulated by ectopic Wnt-1 expression and transactivates the COX-2 promoter (28). Two members of the Wnt-1-induced secreted protein family, WISP-1 and WISP-2, were also identified in our screen. WISP-1 is known to activate the PKB/Akt pathway and block apoptosis (29), and the aberrant expression of WISP-1 may influence the growth of certain tumors (30). Recent evidence suggests that both COX-2 and WISP-1 are strong effectors of Wnt-1-mediated antiapoptosis (14), strengthening the hypothesis that Wnt-1 can induce the expression of several proteins that collectively promote cell survival through the suppression of multiple apoptotic signaling pathways.

MDR1, also induced by Wnt-1, is a glycoprotein that was first identified in Chinese hamster ovary cells. Its expression is associated with the ability of these cells to survive treatment with colchicine (31). Since that early discovery, increased MDR1 expression has been recognized as a hallmark of chemotherapy resistance in transformed cells (32). Appropriately, the regulation of MDR1 expression has immense clinical relevance in the field of cancer treatment. The fact that Wnt-1 confers cellular resistance and survival in response to the chemotoxic drugs vinorelbine and vinblastine has been reported elsewhere (13). The mechanism of this resistance is TCF-4-dependent, suggesting that it is linked to Wnt-1-induced gene expression rather than the direct activation of antiapoptotic pathways by Wnt-1 signaling (13). The possibility that at least some of the effects of Wnt-1 on apoptosis in response to chemotherapy may be related to increased MDR1 expression and active efflux of toxic compounds from cells warrants further investigation.

It has been long recognized that Wnt-1 is a proto-oncogene activated in mouse mammary tissue following infection with mouse mammary tumor virus (5) and also that Wnt-1 has potent effects on other oncogenes to induce transformation of breast tissue (14). Although c-Myc expression can transform cells and enhance cell growth, it also promotes apoptosis in these cells. Expression of Wnt-1 alleviates the effects of c-Myc on apoptosis (14). Furthermore, the Wnt-1-induced genes, COX-2 and WISP-1, are both able to mimic the effects of Wnt-1 on antiapoptosis (14, 30).

Growth factor deprivation can have profound effects on the survival of cells in otherwise nutrient-rich environments (33–35). Furthermore, the regulated balance between cell growth and apoptosis is crucial for the maintenance of tissues (14). A disruption of this balance, leading to either hyperplasia or a loss of programmed cell death, or a combination of the two, is a key step leading to oncogenesis. One focus of cancer research for the last several years has been the discovery of genes that impact normal tissue development and apoptosis. We have presented novel and previously reported genes in which expression is increased by Wnt-1 and which may mediate antiapoptosis in both normal tissue and tumors.

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