Apoptosis of Human Abdominal Preadipocytes Before and After Differentiation Into Adipocytes in Culture

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Differentiation of murine 3T3-L1 preadipocytes into adipocytes is associated with the acquisition of apoptotic resistance accompanied by the upregulation of cell survival genes. We have now examined the effect of adipogenesis on apoptotic susceptibility of human abdominal preadipocytes in primary culture. To induce apoptosis, human preadipocytes, or their differentiated counterparts, were serum-deprived for 24 or 48 hours. When indicated, ceramide was also used as an apoptotic trigger. Cell death was assessed by enumeration of adherent viable cells, and its apoptotic nature was verified by Hoechst staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). After 48 hours of serum withdrawal, cell death was 26% ± 4% in preadipocytes and was increased to 41% ± 4% in differentiated adipocytes (mean ± SE; n = 7 patients; *P* < .002). Under serum-free conditions for 24 hours, ceramide-induced cell death was 40% ± 6% in preadipocytes and increased to 68% ± 8% in adipocytes (mean ± SE; *P* < .01; n = 8 patients). Neuronal apoptosis inhibitor protein (NAIP), an antiapoptotic protein cell survival that increases upon 3T3-L1 adipogenesis, was reduced in human preadipocytes undergoing differentiation (n = 6 patients). Preadipocytes derived from omental versus subcutaneous abdominal fat were more susceptible to apoptosis induced by serum deprivation, 16% ± 4% versus 31% ± 3% cell death, respectively (mean ± SE; *P* < .02; n = 7 patients). Although the murine 3T3-L1 preadipocyte cell line is a useful model that approximates primary preadipocyte cell biology, our data derived from human preadipocyte studies suggest important differences with respect to the regulation of apoptosis.

EXCESS ADIPOSE tissue leads to insulin resistance, thereby increasing the risk of type 2 diabetes mellitus and cardiovascular disease.[1] Adipocytes release a variety of cytokines that can influence energy expenditure, insulin sensitivity, vasomotor tone, and fibrinolysis. Obesity perturbs the regulation of these cytokines. Adipose tissue undergoes dynamic remodeling during adult life. When energy balance is positive, excess calories are stored as triacylglycerol, accomplished through enlargement of adipocytes, as well as through recruitment of committed progenitor cells (preadipocytes) to form more adipocytes.[2] In negative energy balance, lipolysis results in reduced adipocyte volume, and apoptotic reduction in cell number has also been suggested to occur.[3]

Adipose tissue apoptosis has not been extensively investigated. Little is known about either the physiologic regulators of cell death or the apoptotic sensitivity of preadipocytes versus adipocytes. Using the well-established immortalized murine 3T3-L1 cell line model of adipogenesis, we reported that as adipogenesis ensues, these cells acquire resistance to apoptosis induced by growth factor deprivation.[4,5] Expression of 2 cell survival genes, neuronal apoptosis inhibitor protein (NAIP) and Bcl-2, increased, consistent with the differentiation-dependent effect on survival.[5] Others have confirmed the apoptotic sensitivity of 3T3-L1 preadipocytes.[6,7]

Although 3T3-L1 preadipocytes are a valuable experimental model, they do have distinctive attributes compared with human preadipocytes in primary culture, beyond the obvious species difference. 3T3-L1 preadipocytes are aneuploid, as well as embryonal in origin,[8] and such features could influence cell survival pathways. We have now examined the apoptotic responses of human preadipocytes and their differentiated counterparts in primary culture, obtained from abdominal subcutaneous and omental adipose tissue.

MATERIALS AND METHODS

Isolation of Human Preadipocytes

Subcutaneous and omental adipose tissue were obtained from a total of 29 patients (17 women, 12 men) undergoing elective abdominal surgery (approved by the Research Ethics Committee of the Ottawa Health Research Institute). Mean age was 53 ± 2, and mean body mass index was 30 ± 1 (± SE). Preadipocytes were isolated as previously described with minor modifications.[9,10] Briefly, tissue was separated from connective tissue and capillaries by dissection and then digested with collagenase CLS type 1 (200 U/g tissue; Worthington, Lakewood, NJ). The digested tissue was subjected to progressive size filtration and centrifugation, followed by incubation in erythrocyte lysis buffer (155 mmol/L NH_4Cl, 5.7 mmol/L K_2HPO_4, 0.1 mmol/L EDTA, pH 7.3).

Cell Culture of Human Preadipocytes

Preadipocytes were seeded at a density of 3×10^4 cells/cm^2 and grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 50 U/mL nystatin (all from Life Technologies, Burlington, Canada). To induce differentiation, preadipocytes were placed in serum-free medium consisting of DMEM:F12 (1:1), supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 33 μmol/L biotin, 17 μmol/L pantothenate, 10 μg/mL transferrin, 0.2 nmol/L triiodothyronine, 100 nmol/L cortisol, 20 nmol/L insulin, and for the first 4 days, 25 nmol/L dexamethasone, 0.5 mmol/L isobutylmethylxanthine (IBMX), and 5 μmol/L troglitazone (gift from Roche, Laval, Canada).[11] Medium was replaced every 3 to 4 days. After approximately 2 weeks, differentiated adipocytes were either

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lysed and processed for Oil Red O staining or immunoblotting (see below) or placed back in DMEM supplemented with 20% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 50 U/mL nystatin for 48 hours before serum withdrawal for the indicated times. Cell death was induced by incubation in serum-free DMEM supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 50 U/mL nystatin for the indicated times. When indicated, 50 μmol/L C2-ceramide or inactive control C2-dihydroceramide (both from Calbiochem, San Diego, CA) was added to the medium.

Cell Culture of 3T3-L1 Preadipocytes

The 3T3-L1 murine cell line was obtained from American Type Culture Collection (ATCC; Rockville, MD) and maintained in DMEM supplemented with 10% calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. At confluence, cells were serum starved in DMEM supplemented with antibiotics to induce apoptosis for the indicated times as previously described.[5] For differentiation, 2-day postconfluent 3T3-L1 preadipocytes were placed in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 100 nmol/L insulin, and for the first 48 hours only, 0.25 μmol/L dexamethasone and 0.5 mmol/L IBMX.[12] At the end of 8 days, cells were either maintained in or deprived of serum to induce apoptosis, as above.

Oil Red O Staining

Human or 3T3-L1 preadipocytes and their differentiated counterparts were fixed in 10% formaldehyde, washed with phosphate-buffered saline (PBS), and stained with the neutral lipid dye Oil Red O (0.3% in 60% isopropanol), followed by extensive washes.[13] Photomicrographs were generated with an Olympus DP10 camera connected to an Olympus CK2 microscope (Japan).

Cell Enumeration

After the indicated time of serum deprivation, floating cells were removed, and the remaining adherent, viable (>95% by trypan blue exclusion) cells were trypsinized and counted in duplicate on a hemocytometer.[5] Data are expressed as percent cell death according to the following equation [(# adherent cells in serum-fed controls − # remaining adherent cells after treatment)/# adherent cells in serum-fed controls] × 100. Cell death in the serum-fed controls was < 2%.

Hoechst Staining

Human preadipocytes were seeded on glass coverslips, grown to confluence, and induced to differentiate and/or serum starved, as described above. After the indicated treatment, cells were processed for Hoechst staining (Hoechst 33248; Sigma, St Louis, MO) as previously described.[4] Nuclear staining was examined under a Zeiss Axioplan 2 fluorescence microscope (200 × magnification) equipped with an Axiocam camera (Carl Zeiss, Toronto, Canada). The results are expressed as the percentage of apototic cells, ie, the number of apoptotic cells per 100 cells (minimum 500 cells counted) in 10 random fields, performed by 2 independent observers.

TUNEL Assay

Human or 3T3-L1 preadipocytes were seeded on glass coverslips, grown to confluence, and induced to differentiate and/or serum starved, as described above. After the indicated treatment, cells were processed for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using the In Situ Cell Death Detection Kit, AP (Roche), as previously described.[14] Stained cells were visualized with a Nikon TS100 microscope (400 × magnification) equipped with a Coolpix995 camera. The results are expressed as the number of TUNEL-positive cells per 100 cells (minimum 500 cells counted) in 10 random fields performed by 2 independent observers.

Immunoblot Analysis

Human preadipocytes and differentiated adipocytes were lysed in Laemmli buffer.[15] Solubilized protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes, which were then incubated with primary antibodies directed against NAIP (1:1,000; rabbit polyclonal antibody, kindly provided by Drs Z. Yaraghi and A. MacKenzie, University of Ottawa), Bcl-2 (2 μg/mL rabbit polyclonal antibody; Santa Cruz Biotech, Santa Cruz, CA); fatty acid synthase (FAS; 1 μg/mL mouse monoclonal antibody; Transduction Laboratories, Mississauga, Canada), or actin (1 μg/mL rabbit polyclonal antibody; Santa Cruz). After washing, membranes were incubated with the appropriate secondary antibodies (Amersham Pharmacia Biotech, Baie d'Urfie, Canada). Immunoreactivity was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Statistical Analysis

Values are expressed as means ± SE, and groups were compared using Student's *t* test or analysis of variance (ANOVA) with GraphPad Instat version 3.00 (GraphPad Software, San Diego, CA).

RESULTS

The study of human preadipocytes in primary culture is limited due to constraints on preadipocyte yield from adipose tissue, because allowing proliferation of human preadipocytes in culture inhibits subsequent differentiation.[10,16] Recently, thiazolidinedione-based strategies have induced substantial adipogenesis in abdominal subcutaneous preadipocytes, despite limited proliferation and subpassaging[11,17] Using this approach, we were able to isolate and grow human preadipocytes in culture and differentiate them reproducibly and efficiently, with ~75% of preadipocytes converting to the adipose phenotype. This compared favorably with that observed for 3T3-L1 adipogenesis (Fig 1). Adipogenesis was also confirmed by the strong induction of FAS protein in differentiated 3T3-L1 and human adipocytes (Fig 1).

We have reported that as 3T3-L1 preadipocytes differentiate into adipocytes, they acquire apoptotic resistance to serum deprivation.[5] Our current objective was to determine if this was the case for human adipogenesis in primary culture. Abdominal subcutaneous preadipocytes were grown to confluence and then either directly serum deprived (24 hours) or first differentiated into adipocytes followed by serum deprivation (24 hours). Using Hoechst staining to detect apoptotic cells, we observed that the differentiated adipocytes showed higher rates of apoptosis than did preadipocytes (Fig 2). This surprising result prompted us to conduct a rigorous comparison between 3T3-L1 versus human primary preadipocytes (Fig 3).

We first confirmed that 3T3-L1 preadipocytes underwent substantial cell death when placed in serum-free medium for 24 hours, whereas 3T3-L1 adipocytes were significantly less affected (Fig 3A). The apoptotic nature of the cell death and the higher preadipocyte response were confirmed by TUNEL; a shorter period of serum deprivation (3 hours) was used to detect apoptotic cells that remain adherent, as we have described previously[14] (Fig 3C). The same pattern of cell death was observed when troglitazone, used for the human preadipocyte

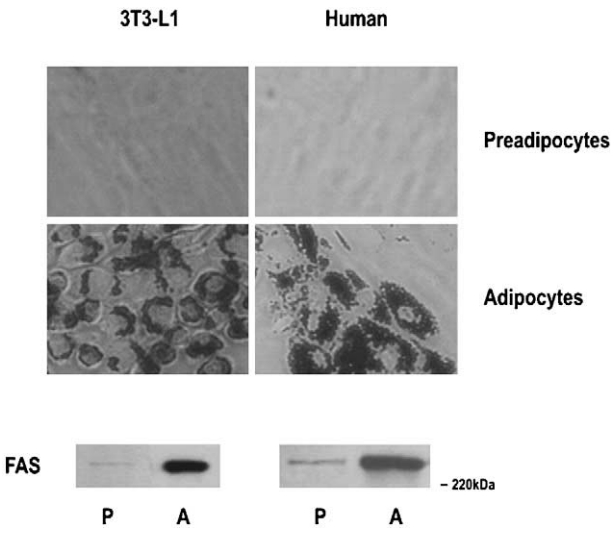

Fig 1. Differentiation of 3T3-L1 and human preadipocytes. Photomicrographs of Oil Red O-stained cultures of 3T3-L1 and human abdominal subcutaneous preadipocytes (P) and differentiated adipocytes (A) are shown. Solubilized protein from corresponding cell cultures was immunoblotted for FAS protein expression, as described.

differentiation, was present in the 3T3-L1 differentiation medium.

Abdominal subcutaneous preadipocytes were grown to confluence and then either directly serum deprived (48 hours) or first differentiated into adipocytes followed by serum deprivation (48 hours). A longer time period of serum deprivation was required to induce cell death compared with 3T3-L1 cells. In direct contrast to the pattern seen with 3T3-L1 cells, the cell death response was much greater in the differentiated human adipocytes than the preadipocytes (41% ±4% *v* 26% ± 4%; n = 7 patients; $P < .002$) (Fig 3B). The individual responses for each of the 7 samples followed this pattern (Fig 3B). TUNEL (Fig 3D) confirmed the apoptotic nature of the cell death, as well as the higher response in human differentiated adipocytes versus preadipocytes (n = 7 patients, $P < .001$). To optimize the detection of TUNEL-positive and adherent preadipocytes undergoing apoptosis before their detachment, the duration of serum starvation was reduced from 48 hours to 16 to 24 hours.

We next investigated whether human preadipocyte differentiation altered the protein expression of NAIP or Bcl-2, both

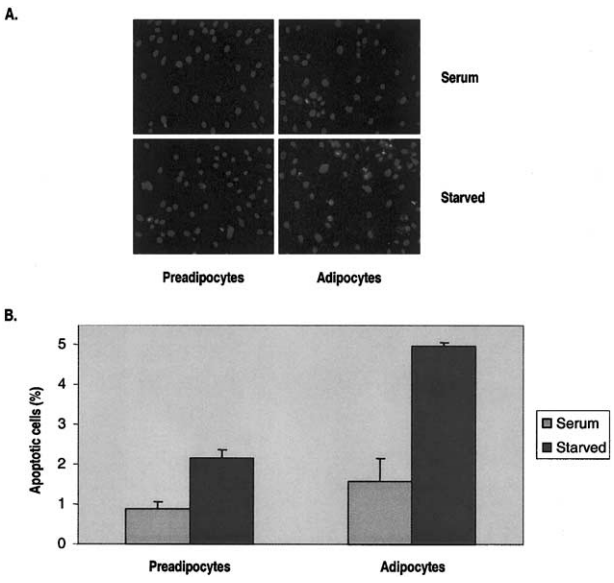

Fig 2. Apoptosis of serum-deprived human preadipocytes and differentiated adipocytes. Cell cultures of human abdominal subcutaneous preadipocytes and differentiated adipocytes were prepared as described. After serum deprivation (24 hours) of cell cultures, Hoechst staining was performed as described. (A) Photomicrographs of cells from a single patient. (B) Data expressed as means ± range, from 2 patient samples.

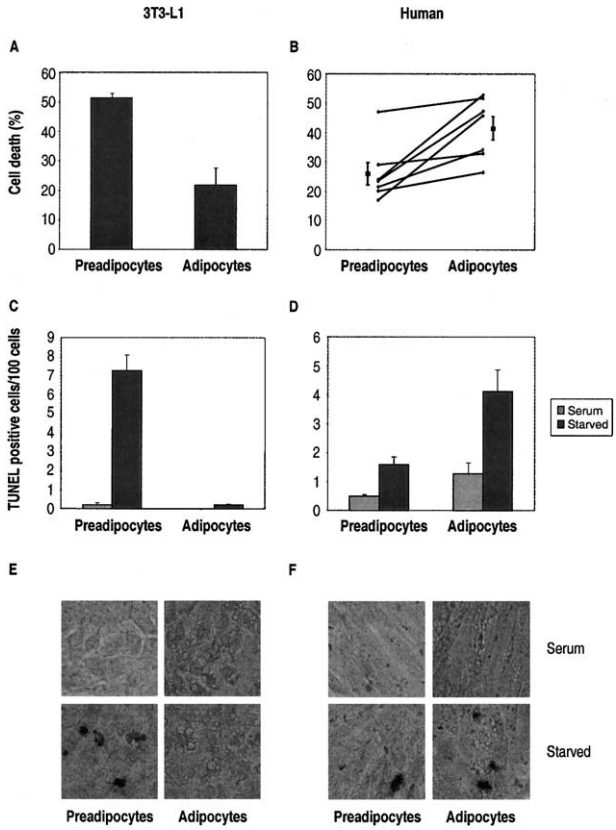

Fig 3. Apoptosis of serum-deprived 3T3-L1 and human preadipocytes and differentiated adipocytes. Cell cultures of 3T3-L1 and human abdominal subcutaneous preadipocytes and differentiated adipocytes were prepared as described. (A and B) After serum deprivation of 3T3-L1 (24 hours) and human (48 hours) cell cultures, cell death rates (means ± SE) were calculated as described. Responses from the 7 individual patient samples are shown (B). (C and D) After serum deprivation of 3T3-L1 (3 hours) and human (16 to 24 hours) cell cultures, TUNEL was performed as described. The data, expressed as means ± SE, are from a total of 7 patient samples, 2 of which were also used for the cell count experiment in (B). (E and F) Representative photomicrographs of 3T3-L1 and human cell cultures maintained in or deprived of serum and then subjected to TUNEL as described.

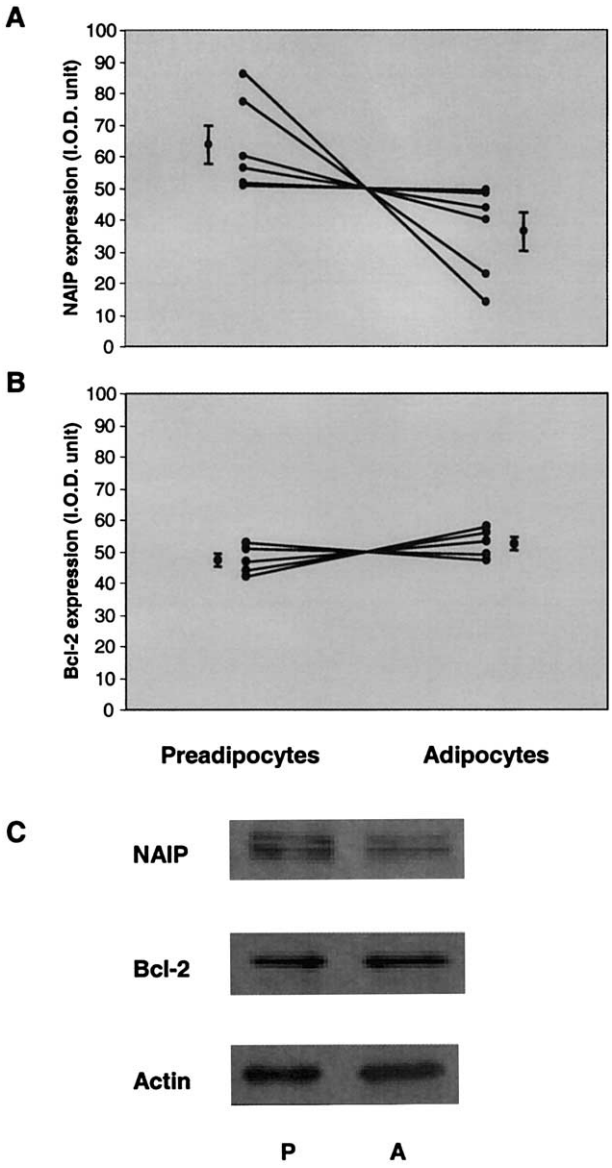

Fig 4. Protein expression of NAIP and Bcl-2. Solubilized protein from human abdominal subcutaneous preadipocytes and differentiated adipocytes (data expressed as means ± SE) were immunoblotted for either NAIP, Bcl-2 , or actin (loading control) protein expression as described. Responses from the individual patient samples are shown in (A) (n = 6) and (B) (n = 5). (C) Representative immunoblots of NAIP, Bcl-2, and actin of one of the patients.

antiapoptotic regulators, which increase during 3T3-L1 adipogenesis. Figure 4 shows that NAIP levels decrease during the differentiation of abdominal subcutaneous preadipocytes (n = 6 patients; $P < .05$). There were no significant differences in Bcl-2 protein during preadipocyte differentiation.

Ceramide has been identified as a bioactive lipid that can trigger apoptosis in several cell types, including preadipocytes.[18] When 50 μmol/L C2-ceramide was added to human abdominal subcutaneous preadipocytes in serum-free medium, cell death was evident by 24 hours (Fig 5A), increasing from 18% ± 2% with serum deprivation alone to 40% ± 6% in the presence of ceramide (mean ± SE, n = 8 patients, $P < .05$). An augmentation in cell death also occurred for differentiated adipocytes in serum-free medium treated with ceramide from 32% ± 3% to 68% ± 8% (mean ± SE; n = 8 patients; $P < .01$). Control inactive C2-dihydroceramide had no significant effect on cell death (Fig 5B). Even in the presence of serum, adipocytes were more sensitive to ceramide with cell death at 8% ± 1% for preadipocytes, and 3-fold higher (22% ± 4%) cell death for differentiated adipocytes (mean ± SE; n = 4 patients, $P < .05$).

Only one previous study has examined depot-specific effects on preadipocyte apoptosis, in which preadipocytes derived from abdominal omental versus subcutaneous depots were found to be more susceptible to apoptosis.[19] We compared apoptosis of abdominal subcutaneous preadipocytes with omental preadipocytes subjected to serum deprivation (Fig 6). Omental versus subcutaneous preadipocytes (mean ± SE; $P < .02$; n = 7 patients) exhibited a greater extent of cell death when placed in serum-free medium for 48 hours (31% ± 3% *v* 16% ± 4%). There were no significant changes in either NAIP or Bcl-2 protein expression between the 2 preadipocyte populations.

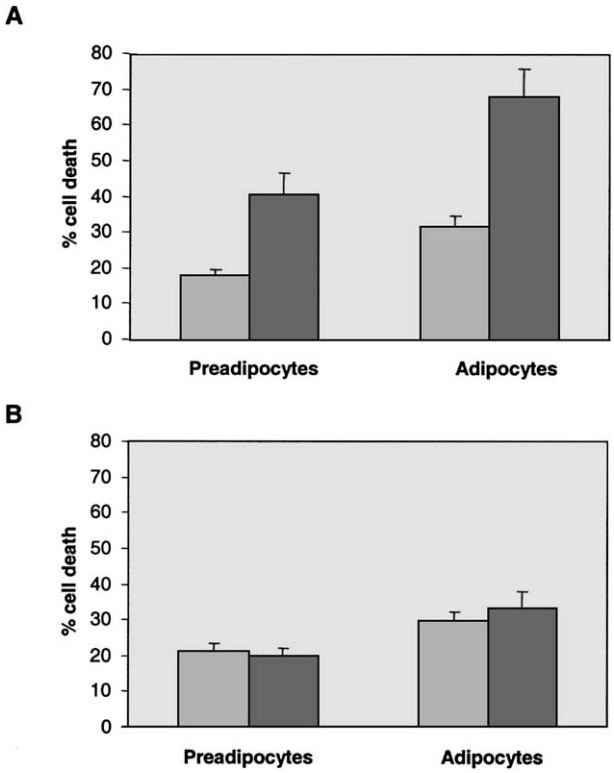

Fig 5. Effect of C2-ceramide on apoptosis. (A) Human abdominal subcutaneous preadipocytes and differentiated adipocytes were assessed for cell death after being deprived of serum in the presence or absence of C2-ceramide (C2; 50 μmol/L) for 24 hours. Data are derived from samples processed from 8 patients and are expressed as means ± SE. (B) The inactive control dihydroceramide (dihydroC2; 50 μmol/L; 24 hours) was used in place of C2. Data are derived from samples processed from 4 patients and are expressed as means ± SE. (▨) Starved; (▩) starved + dihydro C2.

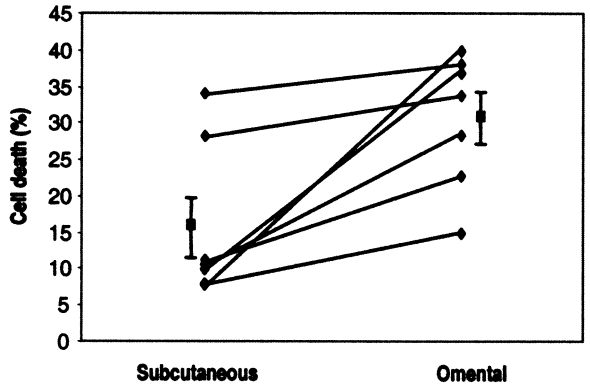

Fig 6. Effect of adipose tissue depot on preadipocyte apoptosis. Preadipocytes, derived from either abdominal subcutaneous or omental adipose tissue depots, were assessed for cell death after 48 hours of serum deprivation. Data are derived from samples processed from 7 patients (individual responses are shown) and are expressed as means ± SE.

DISCUSSION

With respect to the effect of adipogenesis on apoptosis, the behavior of human preadipocytes in primary culture is not accurately reflected by the widely used murine 3T3-L1 preadipocyte model. Our data indicate that human preadipocytes become more sensitive, rather than more resistant, to apoptotic stimuli as they mature into adipocytes in culture.

Murine 3T3-L1 cells have been a mainstay for adipose cell biology research over several decades.[8,20-22] They are an appealing and tractable system, because they respond quite uniformly to hormonal stimuli, proliferate rapidly, and can be cryopreserved and subsequently passaged multiple times without losing responsiveness.

We have previously used 3T3-L1 preadipocytes and differentiated adipocytes to investigate cell survival signaling and the effect of differentiation on apoptosis. 3T3-L1 preadipocytes were found to be more susceptible to apoptosis induced by serum deprivation than were differentiated 3T3-L1 adipocytes.[4] Furthermore, we observed an increase in Bcl-2 and NAIP protein expression that was associated with adipogenesis.[4,5] Others subsequently also reported on the influence of adipogenesis on 3T3-L1 cell apoptosis.[6]

Although murine 3T3-L1 cells are generally a reliable model of human adipogenesis, they do have several differences beyond the obvious one related to species of origin. Immortalized 3T3-L1 preadipocytes are embryonal in origin, are aneuploid, and undergo early clonal expansion during differentiation in culture.[23] Human preadipocytes are isolated from adults, are diploid, and differentiate in culture without a clonal expansion phase.[16,24] Differences in the expression patterns of signal transducer and activator of transcription (STAT) isoforms in 3T3-L1 versus human adipogenesis were also recently reported.[25] Therefore, direct examination of apoptosis in human preadipocytes in culture is prudent to assess the implications of data derived from the 3T3-L1 cell model.

Our studies on human preadipocytes undergoing differentiation into adipocytes are noteworthy, in that apoptotic susceptibility as a function of differentiation is opposite to that of the 3T3-L1 cell line.[4-6] In keeping with this observation, the upregulation of NAIP and Bcl-2 that occurs with 3T3-L1 adipogenesis[4,5] did not occur during human adipogenesis. In fact, consistent with the higher rate of human adipocyte apoptosis, there was a significant reduction NAIP expression; whether NAIP deficiency may have a role to play in the higher apoptotic susceptibility of differentiated human adipocytes awaits future studies specifically aimed at examining this question. The pattern of Bcl-2 expression did not suggest any detectable influence on survival during adipogenesis. Other potential molecular mechanisms for the changes in apoptotic responsiveness during human adipogenesis remain to be defined.

Ceramide is a sphingomyelin-derived lipid whose production can be stimulated by tumor necrosis factor (TNF)α, and which may interfere with growth factor survival signaling in adipocytes.[26,27] It has been reported to trigger apoptosis in differentiated rat preadipocytes.[18] Our data demonstrate that ceramide also induces cell death of human preadipocytes and differentiated adipocytes, in the presence or absence of serum. In either context, the differentiated human adipocytes are more susceptible than preadipocytes to ceramide. The ceramide-induced apoptosis observed by us is consistent with reports that implicate TNFα as an apoptotic agent for human adipose cells, discussed below.

Published studies examining apoptosis in primary cultures of human preadipocytes or adipocytes are limited. Prins et al[28] reported apoptosis in human preadipocytes, assessed by the nuclear morphology of adherent cells using acridine orange. They found that serum deprivation induced apoptosis in up to 2.9% of cells; addition of TNFα increased the response to 4.5%. Parallel studies on preadipocytes and adipocytes differentiated in culture were not performed. A subsequent study from the same group reported on apoptosis, judged by acridine orange staining of adherent cells, in human preadipocytes from abdominal subcutaneous and omental depots.[19] Serum deprivation led to apoptotic changes in 3% and 7% of subcutaneous and omental preadipocytes, respectively. Our study is just the second one to document, and therefore confirm, the greater predisposition of omental versus subcutaneous preadipocytes to apoptosis. We did not detect any significant changes in NAIP or Bcl-2 protein expression to account for this observation. A differential display-based comparison showed a higher level of cellular inhibitor of apoptosis protein-2 mRNA in omental versus subcutaneous adipocytes, but no data were reported for preadipocyte gene expression.[19,29] The potential role of regional site-specific preadipocyte turnover merits further investigation. Although our focus in these experiments was limited to the 2 candidate proteins (NAIP and Bcl-2) that we characterized in our previous studies, a broader-based screening strategy using human preadipocytes from the 2 adipose tissue depots to identify other apoptosis-related proteins should be informative.

Working with isolated human adipocytes and an alternative ceiling culture approach, Zhang et al[30] showed that 30% to 35% of adipocytes undergo apoptosis after serum deprivation versus 10% to 15% in serum-fed control adipocytes. The unusually high baseline rate of apoptosis in the serum-fed control cells in their study was not explained, and so the cellular response to serum deprivation is somewhat difficult to interpret. It is not clear whether this was related to technical aspects of cell

isolation or culture conditions. With our methodology, serum-fed control human adipocytes differentiated in culture had minimal (<2%) apoptosis, indicating that the cells were not under any stress under basline conditions.

Understanding more about the process of adipose cell apoptosis is imperative. It has been proposed as a possible target for future obesity therapeutic strategies,[31] and perturbations in its regulation might contribute to the human immunodeficiency virus (HIV) protease inhibitor-associated and genetic forms of lipoatrophic diabetes.[32,33] Establishing appropriate culture models of adipose apoptosis is a critical first step. Murine adipose cell lines are used widely to study cell death regulation.[34-36] The data presented here indicate that it is critical to consider the response of human preadipocyte/adipocyte culture systems in investigations of adipose cell apoptosis, because conclusions based on murine adipose cell lines may not be accurate models for this process.

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