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Letter to the Editor

White adipocytes are less prone to apoptotic stimuli than brown adipocytes in rodent

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Dear Editor,

Obesity in rodents is related to a functional atrophy of brown adipose tissue (BAT), and consequent impairment of adaptive thermogenesis and increased white adipose tissue (WAT) mass.[1] Further to defects in molecular processes of the adaptive thermogenesis, the BAT atrophy may be due to an increased rate of brown fat cell death.[2] The expression of tumor necrosis factor (TNF)-α is elevated in WAT of a variety of obese animals[3] and humans,[4] and TNF-receptor 1 (TNFR-1) stimulation induces brown adipocyte apoptosis both *in vitro* and *in vivo*.[2,5] Although there is some evidence that apoptosis of white adipocytes may be relevant in certain situations,[6,7] its role in WAT accumulation is not clearly defined. Here, a different sensitivity to apoptotic stimuli is reported in brown and white adipocytes, concomitant with a cell-specific expression of pro- and anti-apoptotic molecules. This difference is evident also in BAT and WAT of rats either subjected to starvation or genetically obese.

Firstly, we found that brown adipocytes, unlike white fat cells, exposed to TNF-α (1 and 10 nM) plus 10 μg/ml cycloheximide (CHX) for either 5 and 24 h, underwent to apoptosis, as evidenced by DNA fragmentation on agarose gel and cytofluorimetric analysis (Figure 1 and Supplementary Figure 1). A marked DNA fragmentation was evident in brown adipocytes, whereas in white adipocytes it was evident only after 4 h and at the highest dose. Both cellular types showed a similar TNR-1 protein level (Supplementary Figure 2), suggesting that the different sensitivity of these two cell types might be due to differences in intracellular apoptotic signaling pathways.

Thus, to investigate these differences, we evaluated the caspase-8 activity in brown and white adipocytes after 10 nM TNF-α plus CHX exposure for a short interval of time (0–180 min). Caspase-8 activity was increased after 15 min of treatment and was maximal after 2 h of treatment in brown adipocytes. In contrast, after 3 h of treatment no significant increase of the enzyme activity was evident in white adipocytes (Supplementary Figure 3a). Moreover, the unprocessed pro-caspase-8 protein levels in brown adipocytes were decreased after 30 min of treatment with a maximum after 3 h, whereas these levels remained unchanged in white adipocytes (Supplementary Figure 3b). Finally, pretreatment of either cells with the specific inhibitor of caspase-8, Z-IETD-FMK (50 nM, 1 h before TNF-α plus CHX treatment, which was prolonged for further 1 or 3 h) confirmed the relevance of caspase-8 in apoptosis of brown adipocytes (Supplementary Figure 3c). The pretreatment with Z-IETD-FMK did not totally block the DNA fragmentation, suggesting that the mitochondrial pathway plays an essential role in triggering apoptosis of brown fat cells.

The active caspase-8 processes Bid to produce truncated Bid (tBid), which in turn mediates the cytochrome *c* release from the mitochondria into the cytosol, where it triggers the activation of caspase-9.[8] The activated caspase-9 induces additional events, including the poly(adenosine diphosphate-ribose) polymerase (PARP) cleavage and DNA fragmentation.[8] Thus, Bid and tBid expression, release of cytochrome *c* and cleavage of PARP were analyzed by means of immunoblotting after 10 nM TNF-α plus CHX exposure of brown and white adipocytes. TNF-α plus CHX treatment time-dependently increased the levels of tBid in brown unlike white adipocytes starting from 30 min with a maximum at 120 min (Supplementary Figure 4a). A very low tBid amount was evident only after 120–180 min of treatment in white adipocytes. Supplementary Figure 4b shows that TNF-α plus CHX treatment increased the cytochrome *c* release from the mitochondria into the cytosol in brown adipocytes after 2 h, unlike white adipocytes in which cytochrome *c* remained in mitochondrial fraction, whereas no signal was evident in the cytosolic one, also after 6 h. The purity of the mitochondrial and cytosolic fractions was tested by means of the mitochondrial membrane cytochrome *c* oxidase (complex IV, COX IV) immunolabeling. Interestingly, cytochrome *c* was also evident in the cytosol of the untreated brown fat cells. Next, the cleavage of PARP was analyzed in a time course experiment (Supplementary Figure 4c), in which the cells were treated as reported. As expected, the presence of cleaved PARP products was evident after 2 h and increased further after 3 h of treatment only in brown, but not in white adipocytes. Finally, Supplementary Figure 4d shows that after 10 nM TNF-α plus CHX exposure caspase-3 activity was increased in a time manner in brown adipocytes, whereas the enzyme activity was not changed significantly in white adipocytes. These results strongly confirm that TNF-α can trigger the apoptotic signaling in brown more than in white adipocytes.

We hypothesized that the resistance to TNF-α-induced apoptosis of white adipocytes could be due to a cell-specific expression of pro- and anti-apoptotic molecules and processes. Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis showed that the Bcl-2/Bax mRNA ratio was higher in white than in brown adipocytes after the TNF-α plus CHX treatment (data not shown). Moreover, Supplementary Figure 5a confirms that the Bcl-2 protein

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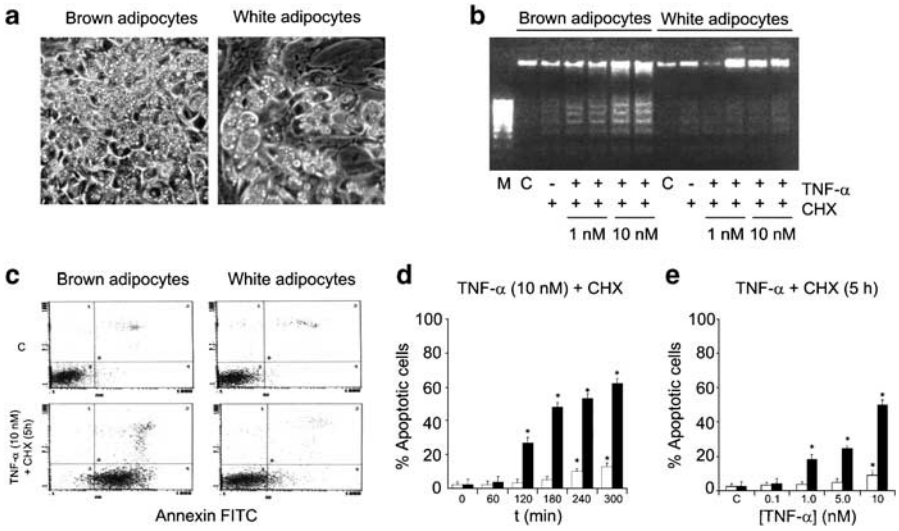

Figure 1 Different grades of apoptosis in brown and white adipocytes in culture. (**a**) Phase-contrast photomicrographs of brown and white adipocytes differentiated in culture. Magnification, $\times 20$. (**b**) DNA fragmentation in cells treated for 5 h with either 1 or 10 nM TNF-α plus CHX. M, molecular marker; C, untreated cells. (**c**) Cytofluorimetric analysis. The cells were treated with 10 nM TNF-α plus CHX for 5 h and analyzed for AnnexinV-FITC positivity: square 2, propidium iodide-positive cells; square 3, nonapoptotic cells; square 4, apoptotic cells. (**d**, **e**) Cytofluorometer analysis of time- and dose-dependence. Open bars, white adipocytes; closed bars, brown adipocytes. The bars represent the mean values $\pm$ S.E.M. of three separate experiments. $^*P < 0.01$: difference from untreated cells (C) at time 0

levels were higher in white than in brown fat cells, whereas the levels of Bax were higher in brown than in white adipocytes at every time point tested. Interestingly, the Bcl-2 protein levels were high in primary mature white adipocytes and in 3T3-L1 differentiated cells (i.e., an adipose cell line that differentiates into white adipocytes) (Supplementary Figure 5b). This observation seems to strengthen that white adipocytes characteristically express high level of Bcl-2. Indeed, also the 3T3-L1 cells differentiated in adipocytes showed a marked resistance to anti-apoptotic stimuli (data not shown).[9]

Apoptotic stimuli can in some circumstances result in increased production of the Bax protein and its subsequent transduction to mitochondria. However, this did not occur after TNF-α plus CHX treatment of both brown and white adipocytes, because we did not detect a marked change in the cellular content of Bax at different time points (Supplementary Figure 5a). We next examined the association of Bax with mitochondrial membrane by immunoblotting of mitochondrial and cytosolic fractions of brown and white adipocytes after TNF-α plus CHX treatment. Bax was found predominantly in the cytosolic fraction of control brown fat cells whereas it was found only in the cytosolic fraction in white adipocytes before treatment (Supplementary Figure 5c). In brown adipocytes exposed to TNF-α plus CHX for different time intervals, a significant amount of Bax was recovered also in the mitochondrial fraction (Supplementary Figure 5c). Conversely, Bax remained associated with the cytosolic fraction of white adipocytes after TNF-α plus CHX treatment (Supplementary Figure 5c).

Remarkably, under our experimental conditions, 8–24 h serum deprivation induced a marked apoptosis measured as DNA fragmentation and cytofluorometric analysis only in brown adipocytes, but not in white adipocytes (Supplementary Figure 6).

The higher sensitivity of brown than white adipocytes to apoptotic stimuli was observed also *in vivo*. We showed that Bcl-2 protein levels were markedly lower in BAT of obese Zucker *fa/fa*, in which TNF-α is overexpressed,[3] than in lean control rats.[10] Conversely, the high Bcl-2 expression level in white fat of control rats was unchanged in the obese animals. Moreover, the expression of several proapoptotic genes, including Bax, Bad, Bak, and Bid, was markedly higher in BAT of obese as compared to lean control animals. Moreover, even if Bad, Bak, and Bid mRNA levels were higher also in WAT of obese as compared to lean rats, the Bax gene expression was unchanged (Supplementary Figure 7). Furthermore, in rats kept at room temperature for 48–72 h without food, nearly all the WAT depots disappear.[11] However, no apoptotic cells were visualized by electron microscope in the retroperitoneal white fat depot, which was characterized by extreme shrinkage after starvation (data not shown).

In this study, we have demonstrated that white adipocytes are less prone to apoptotic stimuli, including *in vitro* TNF-α exposure and serum deprivation, and *in vivo* starvation, than brown adipocytes. A relevant finding was the high expression level of the anti-apoptotic Bcl-2 protein in white adipocytes when compared to brown fat cells either in unstimulated conditions or after exposure to TNF-α plus CHX. Interestingly, the levels of Bcl-2 have been observed to increase during 3T3-L1 adipogenesis,[9,12] which was accompanied by an increased resistance to apoptosis.[9] Noteworthy, the death signaling pathway (i.e., the caspase-8-induced Bid cleavage to the active tBid, with Bax activation, cytochrome *c* release and PARP cleavage, that in turn activate the major execution caspase, caspase-3) was triggered specifically and markedly in brown adipocytes.

Consistent with the marked resistance to apoptosis of white adipocytes, we were unable to detect any apoptotic white

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adipocytes in WAT of both control and obese *fa/fa* rats, which were characterized by high levels of Bcl-2 (data not shown),[2] and in different fat depots of rats starved for 2–3 days. Consistently, also after serum deprivation the apoptosis was really evident only in brown but not in white fat cells. Moreover, also during lactation, when the WAT mammary gland is substituted by epithelial secretory cells, another situation in which fat apoptosis could be hypothesized, no apoptotic signs were evident in mammary adipose tissue.[13]

Even if apoptosis of white adipocytes has been observed in human explants subjected to growth factor deprivation, elevated temperature, or TNF-α exposure,[7,14] the apoptotic indexes were low (5–25% of total cells). Thus, the relevance of apoptosis in WAT remains extremely controversial. Our present results strongly suggest that white adipocytes are more resistant to apoptotic stimuli both under *in vitro* and *in vivo* experimental conditions. However, they do not exclude a physiological or pathophysiological relevance of apoptosis in WAT.[15,16]

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)