Adipose-Derived Mesenchymal Stem Cells as Stable Source of Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Delivery for Cancer Therapy

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Abstract
Adipose-derived mesenchymal stromal/stem cells (AD-MSC) may offer efficient tools for cell-based gene therapy approaches. In this study, we evaluated whether AD-MSC could deliver proapoptotic molecules for cancer treatment. Human AD-MSCs were isolated and transduced with a retroviral vector encoding full-length human tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a proapoptotic ligand that induces apoptosis in a variety of human cancers but not normal tissues. Although several studies have documented the antitumor activity of recombinant human TRAIL, its use in vivo is limited by a short half-life in plasma due to a rapid clearance by the kidney. We found that these limitations can be overcome using stably transduced AD-MSC, which could serve as a constant source of TRAIL production. AD-MSC armed with TRAIL targeted a variety of tumor cell lines in vitro, including human cervical carcinoma, pancreatic cancer, colon cancer, and, in combination with bortezomib, TRAIL-resistant breast cancer cells. Killing activity was associated with activation of caspase-8 as expected. When injected i.v. or s.c. into mice, AD-MSC armed with TRAIL localized into tumors and mediated apoptosis without significant apparent toxicities to normal tissues. Collectively, our results provide preclinical support for a model of TRAIL-based cancer therapy relying on the use of adipose-derived mesenchymal progenitors as cellular vectors. Cancer Res; 70(9); 3718-29. ©2010 AACR.

Introduction
Mesenchymal stromal/stem cells (MSC) have gained interest as promising tools for cancer therapy because wild-type (WT) and gene-modified (GM) bone marrow (BM) MSC may exert an antitumor potential (1–6). Whereas BM has been the first recognized source of MSC (7), adipose tissue represents a valid reservoir of mesenchymal progenitors (8). Adipose tissue can be obtained in relevant amount and easily processed to release large numbers of adipose-derived MSC (AD-MSC; refs. 9, 10). Similarly to BM-MSC, AD-MSCs are particularly suitable for cell gene therapy approaches because they can be expanded and then transformed by several vectors (11–13).

Starting from this background, we wanted to test whether AD-MSC could represent an efficient vehicle to deliver tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL). TRAIL is a promising anticancer death ligand with a sequence homology to TNF and FasL (14). It is a type II membrane-bound (MB) protein that can be processed by cysteine protease to generate a soluble ligand (15). Both MB protein and soluble ligand can rapidly induce apoptosis in a variety of cancers, sparing normal cells (16). TRAIL mediates the apoptotic effect binding to its death receptors (DR), as homotrimer, particularly on TRAIL-R1/DR4 and TRAIL-R2/DR5 activation, a protein complex, causes caspase-8 activation, triggering apoptosis (17–19). Although several studies have shown the antitumor activity of recombinant TRAIL (rTRAIL), its in vivo use is limited due to short half-life in plasma (20). To overcome these limitations, we generate stably modified AD-MSC to obtain cellular vehicles for a targeted and constant TRAIL delivery. We here originally describe the efficiency of human AD-MSC armed with TRAIL to induce apoptosis in several tumor types and, particularly, in a human cervical carcinoma model.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-09-1865
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Materials and Methods

Cell lines and primary tumor cells. HeLa cells (American Type Culture Collection, LGC srl) were cultivated in DMEM (Life Technologies) with 10% fetal bovine serum (Hyclone), 1% glutamine (200 mM/L), and 1% penicillin-streptomycin (10,000 μg/mL and 10 mg/mL; Euroclone). Primary tumor specimens were obtained after informed consent from patients with histologically documented lung cancer. Tumor cell suspensions were obtained as previously reported (21). For morphologic analyses, trypsinized cells were spun onto slide and stained by standard H&E staining.

Isolation of TRAIL cDNA, vector production, and AD-MSC transduction. Full-length human TRAIL gene (NM_003810.2) was amplified from cDNA isolated by Expand High Fidelity Taq (Roche), as described (22). The following primers containing XhoI and EcoRI sites were used: 5'-ATGGCTATGATGGAGATCTCA-3' (forward) and 5'-CCCCCGAAAATCACCAGATT-3' (reverse). A bacitracin murine stem cell virus-derived retroviral vector (pMIGR1) encoding for green fluorescent protein (GFP) was modified, including the amplified full-length human TRAIL cDNA. The resulting vector was defined as MIGR1-TRAIL-GFP, whereas the empty MIGR1-GFP vector was used as control. Retrovirus production was performed by the FLYR18 packaging cell lines, as published (23). After approval by local Ethical Committee, AD-MSCs were obtained from individuals performing liposuction for aesthetic purposes and processed as published (23). After approval by local Ethical Committee, AD-MSCs were obtained from individuals performing liposuction for aesthetic purposes and processed as published (23). After approval by local Ethical Committee, AD-MSCs were obtained from individuals performing liposuction for aesthetic purposes and processed as published (23).

Fluorescence-activated cell sorting analysis. Cells were stained with PE-anti-TRAIL, PE-anti-TRAIL-R1/DR4, PE-anti-TRAIL-R2/DR5 (Biolegend), and isotype controls (Becton Dickinson). Intracellular staining on transduced AD-MSC and controls was performed with BD Cytofix/Cytoperm kit (Becton Dickinson) using the PE-anti-TRAIL antibody.

ELISA. TRAIL was measured by Quantikine Human TRAIL/TNFsf10 kit (R&D Systems) according to instructions.

Apoptosis and caspase-8 activation assays. HeLa cells were seeded, and after 12 h, either AD-MSC TRAIL or AD-MSC GFP were added at different T:E ratios (1:1, 1:2, and 1:5). Activity of AD-MSC TRAIL was evaluated by propidium iodide (PI; Sigma) staining after 24 and 48 h by fluorescence-activated cell sorting (FACS) gating on GFP-negative cells. Soluble rTRAIL (up to 20 μg/mL; PeproTech, Inc.) was used as positive control. Experiments were performed at least thrice. Isolated primary lung tumor cells were cocultured with either AD-MSC TRAIL or AD-MSC GFP at 1:5 T:E ratio, and tumor cell death was assessed as described above.

Caspase-8 activation was measured by FACS with the CaspGLOW Red Active Caspase-8 Staining kit (Biovision Research, Inc.) coculturing either AD-MSC TRAIL or AD-MSC GFP with HeLa (T:E ratio: 1.5 for 8 h) using as negative control the caspase inhibitor Z-VAD-FMK (10 μM). In addition, cocultures of HeLa cells with either AD-MSC TRAIL or AD-MSC GFP were established using a 0.4-μm Transwell system (Corning, Inc.) to separate the two populations (T:E ratios: 1:1, 1:2, and 1:5). For inhibition studies, cocultures of HeLa cells and AD-MSC TRAIL (T:E ratio: 1:1) were treated with 0.8, 1.6, 3.2, and 6.4 μg/mL of neutralizing anti-human TRAIL antibody (PeproTech). HeLa viability was tested by FACS after PI staining.

Animal studies. Eight- to 10-wk-old NOD.CB17-Prkdcsid/j male and female mice (Charles River) were kept in accordance with guidelines under approved protocols. Six groups of mice (n ≥ 3/each) were considered as follows: (a) s.c. flank injected once with 1 × 106 AD-MSC GFP; (b) s.c. flank injected once with 1 × 106 AD-MSC TRAIL; (c) s.c. flank injected once with 2 × 105 HeLa; (d) s.c. flank injected with 2 × 105 HeLa, and, as soon as an appreciable tumor burden appeared (15–20 d), treated with multiple (n = 3) biweekly intratumor injections of 105 AD-MSC GFP; (e) tumor injected as in (d) but treated with multiple (n = 3) biweekly intratumor injections of 105 AD-MSC TRAIL; (f) tumor injected as in (d) but treated with multiple (n = 3) biweekly tail i.v. injections of 105 AD-MSC TRAIL; (g) tumor injected as in (d) but treated with multiple (n = 3) biweekly tail i.v. injections of 105 AD-MSC TRAIL.

AD-MSC TRAIL toxicity was assessed in groups (a) and (b), particularly considering liver (24). Parameters such as survival, weight, and serum liver enzymes by spectrophotometer (Cobas C501, Roche Diagnostic) were recorded. In groups (c) to (g), weights were weekly measured and tumor sizes were calculated as reported (25): volume = length × width2/2. After 60 d, animals were sacrificed, liver was harvested, and tumor was excised

PCR. GFP-marked AD-MSCs were monitored in excised and processed tumors by PCR using GFP (5'-GTAAAGCCGCCAACAATTCAG-3' and 5'-TGTCGAGCATATAGACG-3'; DQ768212) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5'-GCAGTGGCAAAATGGAGATT-3' and 5'-GCAAGGGGCGAGATG-3'; XM_973383) primer pairs.

Histology. Histochemistry was performed as reported (26).

Statistics. Data are expressed as the mean ± SD. A two-tailed P value of ≤ 0.05 from Student’s t test was considered statistically significant by Excel 2003 software (Microsoft, Inc.).

Results

AD-MSC can be genetically modified to express high levels of TRAIL. AD-MSCs were transduced by vector encoding for full-length human TRAIL (MIGR1-TRAIL-GFP) and with control vector (MIGR1-GFP). In both cases, >95% of GM AD-MSCs were obtained (Fig. 1A, left column). FACS analyses show that WT AD-MSC and AD-MSC GFP do not constitutively express TRAIL; in contrast, gene modification of AD-MSC with TRAIL-encoding vector allows a relevant protein expression by surface and intracellular stainings (Fig. 1A, middle and right columns). Because TRAIL in nature can be released as soluble ligand (14), we wanted to evaluate whether this soluble form could be also produced by AD-MSC TRAIL. A time course experiment (Fig. 1B), started with confluent culture of...
AD-MSC TRAIL, allows to detect soluble TRAIL starting from 6 hours (126.8 ± 18.4 pg/mL), and further analyses at 12, 24, and 48 hours show a constant release of soluble TRAIL (up to 366.4 pg/mL).

Having shown that AD-MSC TRAIL express the desired protein, we evaluated whether forced TRAIL production could be followed by death of AD-MSC themselves. Thus, we investigated TRAIL-R1/DR4 and TRAIL-R2/DR5 expression on WT AD-MSC, uncovering that these cells lack TRAIL-R1/DR4 and show low (<26%) TRAIL-R2/DR5 (Fig. 1C). These findings were validated by PI staining (at 24 and 48 hours), which reveals no differences (P > 0.08) in cell death between confluent WT AD-MSC, AD-MSC GFP, and AD-MSC TRAIL (Fig. 1D). Annexin V staining performed to detect early apoptosis (Supplementary Fig. S1A) further indicates that ~85 ± 4% of AD-MSCs are refractory to TRAIL expression, prompting their use in our cancer gene therapy approach.

**AD-MSCs are not affected by retrovirus transduction and TRAIL expression.** AD-MSCs were then analyzed for known surface antigens and differentiation potentials (27). As shown in Supplementary Fig. S2A, WT and GM AD-MSC express high level of CD90, CD105, CD73 lacking of CD45, CD34, and CD14. In addition, adipogenic and osteogenic differentiation assays indicate that gene modifications do not affect main AD-MSC differentiation pathways (Supplementary Figs. S2B and C and S3A and B). Collectively, these data indicate that the mentioned cell manipulations do not perturb the main AD-MSC features.

**AD-MSCs expressing TRAIL display an in vitro antitumor activity in cancer cell lines.** HeLa cells have been
selected by FACS according to their high expression of TRAIL-R1/DR4 and TRAIL-R2/DR5 receptors as predictive markers for TRAIL sensitivity (Fig. 2A). Moreover, a concentration-dependent effect of rTRAIL on HeLa has been tested to optimize their sensitivity (Supplementary Fig. S1B).

Coculture experiments were then performed; at 24 hours, AD-MSC TRAIL induce HeLa death represented by reduction of adherent HeLa cells and the appearance of cellular debris. These features are even more prominent at 48 hours (Fig. 2B, arrows). The native fluorescence of transduced AD-MSC allows a clear distinction between target and effector cells either by fluorescence microscopy (Fig. 2B) or by FACS (Fig. 2C). Gating on GFP-negative cells (HeLa), we are able to detect relevant amount (≥70%) of PI-positive cells only in coculture where AD-MSC TRAIL are present (Fig. 2C, bottom). To quantify cell death at 24 and 48 hours, different T:E ratios were assessed using AD-MSC TRAIL and AD-MSC GFP as control. As seen in Fig. 2D, cytotoxicity on HeLa cells is detectable only in coculture with TRAIL-producing AD-MSC starting from the 1:1 ratio (P < 0.01). At 24 hours, this effect increases significantly with lower T:E ratios (P < 0.003) and is even more prominent than rTRAIL (P < 0.002). Interestingly, cytotoxicity persists up to 48 hours and is marginally affected by an increase of AD-MSC TRAIL number (P > 0.22). For all the considered ratios, AD-MSC GFP do not cause HeLa death, indicating a specific action of AD-MSC TRAIL.

Having established optimal coculture conditions of our gene therapy approach, we wanted to test other two cell lines known to be sensitive to rTRAIL (28, 29). Thus, pancreas (BxPc3) and colon adenocarcinomas (LS174T) were cocultured with AD-MSC TRAIL using AD-MSC GFP and rTRAIL as controls (Supplementary Fig. S4A and B). At 24 and 48 hours, AD-MSC TRAIL are able to induce a comparable or even superior apoptosis than rTRAIL (20 μg/mL). In addition, analogous approaches were performed on BT549 (breast cancer) and IMR32 (neuroblastoma), known rTRAIL-resistant cell lines (30, 31). As expected, AD-MSC TRAIL were unable to induce a significant apoptosis, underlining the high specificity of this strategy (Supplementary Fig. S4A and B).

**AD-MSC TRAIL specifically induce apoptosis by cell-to-cell contact through caspase-8 activation.** To confirm that HeLa mortality is specifically due to antitumor effect of TRAIL-expressing AD-MSC, anti-TRAIL antibody was diluted in coculture media (Fig. 3A). Starting from 1.6 μg/mL, we observe a significant (P < 0.02) reduction of HeLa cell death, and this effect is more prominent at the highest concentration (6.4 μg/mL) where HeLa death is comparable with control (P = 0.08).

To additionally validate at the intracellular level the specificity of TRAIL effect, we investigate caspase-8 activation. HeLa cells were cocultured with either AD-MSC TRAIL or AD-MSC GFP at 1:5 ratio for 8 hours both in the presence and in the absence of a known caspase inhibitor, Z-VAD-FMK (32). Gating on GFP-negative cells, we are able to identify and selectively analyze the amount of caspase activation in HeLa cells only. As shown in Fig. 3B (left, bottom), the majority (78.3 ± 2.5%) of HeLa cells reveal an activated caspase-8 after 8 hours of coculture with AD-MSC TRAIL (black line), whereas caspase-8 activation is undetectable in the presence of the caspase inhibitor (Fig. 3B, left, bottom, gray line; P = 0.005). On the contrary, either in the presence or in the absence of the inhibitor, caspase-8 activation is undetectable in coculture with AD-MSC GFP (Fig. 3B, left, top; P = 0.003).

We have previously shown that AD-MSC TRAIL express this molecule both as MB protein (Fig. 1A) and as soluble ligand (Fig. 1B). Because both TRAIL forms can exert an antitumoral activity (14), we sought to evaluate the mechanism by which AD-MSC armed with TRAIL could mediate their apoptotic effect. Thus, an in vitro coculture assay has been established using a Transwell system preventing cell-to-cell contact so that HeLa cells, separated from AD-MSC TRAIL, could be induced to death exclusively by soluble TRAIL. Although apoptosis can still be detected in the AD-MSC TRAIL coculture at 1:5 ratio versus HeLa cells alone (P = 0.01), the lack of contact significantly reduces the antitumoral activity by AD-MSC TRAIL (P > 0.06), indicating the cell-to-cell contact as the main mechanism by which AD-MSC TRAIL induce apoptosis (Fig. 3C).

**Bortezomib sensitizes a TRAIL-resistant breast cancer cell line to AD-MSC TRAIL.** Bortezomib cooperates with rTRAIL to induce apoptosis in TRAIL-resistant tumors mainly by upregulation of TRAIL receptors (33). To investigate whether bortezomib could be combined with AD-MSC TRAIL, we selected BT549 as cell line resistant to both rTRAIL and TRAIL cell delivery (Supplementary Fig. S4). We confirmed that BT549 can survive up to 20 μg/mL of rTRAIL (Supplementary Fig. S5A) and lack of TRAIL-R1/DR4 and TRAIL-R2/DR5 (Supplementary Fig. S5B, top). We then uncovered that a 12-hour treatment with bortezomib (10 nmol/L) induces a significant increase of TRAIL-R2/DR5 (P = 0.02), with no significant effect on TRAIL-R1/DR4 (Supplementary Fig. S5B, bottom). We also asked whether bortezomib could upregulate TRAIL-R2/DR5 expression in AD-MSC, inducing their death with negative effect on possible combinations of drug and cell therapy. Interestingly, bortezomib upregulates TRAIL-R2 receptor (from 20% up to 73% after 24 hours of treatment; P = 0.01), and this change is followed by an increase in cell death versus WT AD-MSC (P = 0.02; Supplementary Fig. S5C and D). Nevertheless, the majority (>85%) of AD-MSC TRAIL survive to the treatment (Supplementary Fig. S5D).

Having considered these findings, BT549 cells were treated with bortezomib and cocultured with AD-MSC TRAIL. At 48 hours, the combination of AD-MSC TRAIL and bortezomib versus bortezomib alone (Supplementary Fig. S5E) significantly increases apoptosis (P = 0.01), indicating that bortezomib can be successfully combined with a cell-based TRAIL delivery for resistant tumor cells.

**AD-MSC TRAIL induce apoptosis in primary tumor cells.** To further challenge our cell therapy approach in a primary tumor model, we focused on lung cancer as leading cause of deaths (34). Four distinct lung tumor specimens were processed. Of those, the best *in vitro* performance was observed in cells obtained from a patient affected by signet ring
Figure 2. AD-MSCs producing TRAIL exert a potent cytotoxic effect on a target tumor cell line. A, FACS analyses of TRAIL receptor (TRAIL-R1/DR4 and TRAIL-R2/DR5) expression on HeLa cells. B, in vitro cultures of HeLa alone as control (CTRL; top), HeLa with AD-MSC GFP (middle), and AD-MSC TRAIL (bottom) visualized by both phase-contrast and GFP filter fluorescence microscopy at 1:5 T:E ratio. Scale bar, 200 μm. At 24 h, AD-MSC TRAIL exert a cytotoxic effect on HeLa cells as shown by the presence of cellular debris in culture medium (left; arrows), and this effect is even more prominent at 48 h (right). C, representative FACS plot of PI staining detecting cell death induced by AD-MSC TRAIL at 48 h. Apoptotic cells were identified gating on (red arrow) GFP-negative cells (HeLa). D, cell death by PI staining on gated GFP-negative HeLa cells in coculture with AD-MSC TRAIL, AD-MSC GFP, rTRAIL, and controls. Different T:E ratios have been tested showing significant cytotoxic effects of AD-MSC TRAIL versus AD-MSC GFP both at 24 and 48 h (P < 0.016). Cytotoxicity is maintained up to 48 h (right) when the lowest ratio (1:5) is comparable with rTRAIL at 20 μg/mL (P = 0.9).
Figure 3. AD-MSC TRAIL specifically induce apoptosis by caspase-8 activation through cell-to-cell contact. A, neutralization of TRAIL-induced apoptosis by anti-human TRAIL antibody diluted in cocultures of HeLa cells and AD-MSC TRAIL at 1:1 T:E ratio. HeLa viability is assayed by PI staining. Untreated HeLa cells were used as control. B, FACS analyses at 8 h of caspase-8 activation on gated GFP-negative HeLa cells in coculture with AD-MSC TRAIL and AD-MSC GFP as control (1:5; T:E). Left, bottom, representative histogram showing the caspase-8 activation on HeLa cells after AD-MSC TRAIL coculture (black line). The addition of specific caspase-8 inhibitor (Z-VAD-FMK, 10 μmol/L) counteracts TRAIL activity inhibiting caspase-8 activation (gray line). Top, caspase-8 activation is undetectable also in coculture of HeLa cells and AD-MSC GFP with or without caspase inhibitor. Columns, mean percentage of caspase-8 activation on HeLa cells in coculture (1:5 ratio) with AD-MSC TRAIL or AD-MSC GFP both with (w) and without (w/o) specific inhibitors (P = 0.003); bars, SD. C, HeLa cells were cocultured in Transwell (TW) plates with either AD-MSC GFP or AD-MSC TRAIL and tested for apoptosis by PI staining.

cell carcinoma (SRCC). The histologic staining of the starting tumor specimen typically revealed the positivity for TTF-1, CK7, and the lack of CDX2 and CK20 (data not shown; refs. 35, 36). Starting from 4 days after isolation, SRCC in vitro generated cell clusters that rapidly reached the confluence (Fig. 4A, left and middle). Tumor population was constituted by elements with the classic signet ring jeweler aspect characterized by abundant intracellular mucin and a crescentic nucleus displaced at the cell periphery, as displayed by H&E staining of cultured cells (Fig. 4A, right).

We first investigated the presence of TRAIL receptors on primary SRCC population revealing the exclusive presence
of TRAIL-R2 on the SRCC surface (Fig. 4B). Coculture experiments were then performed with GM AD-MSC at 1:5 T:E ratio at both 24 and 48 hours, obtaining a significant amount (up to 59.9%; \( P < 0.005 \)) of PI-positive cells in coculture with AD-MSC TRAIL versus AD-MSC GFP (Fig. 4C). This effect resulted even more prominent than the one obtained with 20 \( \mu \)g/mL of rTRAIL (\( P < 0.001 \)), suggesting that, even on primary cancer cells, TRAIL delivery by AD-MSC may be more effective than the use of the recombinant protein.

**In vivo toxicology of the AD-MSC TRAIL approach.** Because TRAIL exposure and MSC gene manipulation were associated to side effects (24, 37, 38), we established an *in vivo* toxicology approach assessing the effect of AD-MSC TRAIL. Firstly, based on the reported TRAIL liver toxicity, mice were s.c. injected once into the flank with 1 \( \times \) 10\(^6\) AD-MSC TRAIL or with 1 \( \times \) 10\(^6\) AD-MSC GFP alone, and serum level of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was monitored. As seen in Fig. 5A, both AST (21 ± 1 IU/L and 63 ± 29 IU/L) and ALT (6 ± 1 IU/L and 19 ± 10 IU/L) levels were between ranges (39, 40) in both AD-MSC GFP and AD-MSC TRAIL groups. These data are confirmed by H&E staining (Fig. 5B), where there is no evidence of abnormal hepatocytes and inflammatory cell infiltration. In addition, to verify whether i.v. and s.c. multiple \((n = 3; 1 \times 10^6/each)\) AD-MSC TRAIL and AD-MSC GFP injections might have been linked to toxicity, same tests were repeated, confirming the lack of liver damage (data not shown). To monitor the overall status of treated mice, weight, food intake, and behavior were monitored. As seen in Fig. 5C, both single (left) and multiple s.c. (middle) and i.v. (right) injections of AD-MSC TRAIL or AD-MSC GFP are not affecting animal weight gain (\( P > 0.63 \)). Similarly, food intake and behavior did not reveal pathologic status and necropsies, performed at 60 days, did not provide signs of abnormal GM AD-MSC proliferation.

**AD-MSC TRAIL exert an antitumor activity in vivo.** To validate *in vitro* findings and having considered *in vivo*

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**Figure 4.** AD-MSCs armed with TRAIL induce apoptosis in lung primary tumor cells. **A,** *in vitro* expanded primary lung SRCC observed at 4 d (left) and 7 d (middle) of culture. Scale bar, 200 \( \mu \)m. Right, H&E staining of isolated tumor cells. Scale bar, 400 \( \mu \)m. **B,** FACS analyses of TRAIL-R1 and TRAIL-R2 expression on primary SRCC. Gray, isotypic control. **C,** columns, mean of cell death at 24 and 48 h by PI staining on gated GFP-negative primary SRCC in cocultures with AD-MSC TRAIL or AD-MSC GFP at 1:5 T:E ratio; bars, SD. *, \( P = 0.005 \); **, \( P = 0.00009 \). rTRAIL (20 \( \mu \)g/mL) is used as positive control and SRCC alone represents a negative control.
toxicology, xenotransplant models of cervical carcinoma have been established s.c. transplanting 2 × 10^5 HeLa cells. Tumor burdens started to appear between 15 and 20 days from the inoculum and three doses of AD-MSC TRAIL or AD-MSC GFP (1 × 10^6 cells/each) were injected. AD-MSC TRAIL inhibit tumor cell growth (P < 0.006); on the contrary, both AD-MSC GFP and HeLa alone generate large tumors (Fig. 6A). Similarly, animals treated with multiple (n = 3) i.v. injections of AD-MSC TRAIL (1 × 10^6 cells/each; Fig. 6B) were associated to a reduced tumor burden (P = 0.01). Interestingly, early phases (up to 45 days) after AD-MSC GFP injection have been associated to greater tumor size versus HeLa cells alone. However, at 60 days, this difference disappeared in both s.c. and i.v. injected mice. To validate that tumor growth reduction was due to the presence of injected AD-MSC TRAIL, we were able to detect these cells in tumor samples (Fig. 6C), and to confirm this finding, immunohistochemical analyses performed on tumor sections reveal specific AD-MSC TRAIL localization (Fig. 6D).

Discussion

We here show that GM AD-MSC can be used as a powerful tool in cancer therapy to counteract cancer growth. Others reported the use of BM and cord blood MSC as drug delivery systems (1, 2, 41–47). Dealing with AD-MSC, these cells have been recently used as cancer therapy tools to vehicle prodrug-converting enzyme (13); however, to our knowledge, our strategy represents the very first example of cancer gene therapy based on AD-MSC directly producing a potent pro-apoptotic agent, such as TRAIL.

Adipose tissue has been selected as source of MSC based on a standardized and minimally invasive procedure from normal subjects and from cancer patients, allowing their use in a hypothetical autologous setting.

To begin with, we show that AD-MSCs without gene modification do not constitutively produce TRAIL; second, we excluded the presence of DR on AD-MSC, which could affect cell survival after TRAIL autocrine production. In particular, we could not detect TRAIL-R1/DR4 and low level of TRAIL-R2/DR5 similarly to what has been described on both BM and amnion-derived MSC (48). Taken together, these data originally indicate that human AD-MSC could be an ideal vehicle to delivery TRAIL.

Because rTRAIL potential is known as well as its capability to selectively induce death on tumor cells, sparing...
Figure 6. In vivo antitumor effect of AD-MSC TRAIL. A, tumor inhibition by AD-MSC TRAIL s.c. injected. A significant reduction of tumor burden is detected in mice treated with AD-MSC producing TRAIL in comparison with both HeLa cells only and AD-MSC GFP.*; \( P = 0.006; **; \( P = 0.002. 

Right, representative tumor specimens taken from a HeLa cell–injected mouse and mouse treated with HeLa and AD-MSC TRAIL. B, AD-MSC TRAIL i.v. injections reduce tumor growth in treated mice. *, \( P = 0.01. 

C, reporter gene (GFP) amplification in tumors taken from mice treated with AD-MSC TRAIL. The GFP plasmid is the positive control (PC). MK, marker; NC, negative control. GAPDH is used as housekeeping gene. D, representative photomicrographs of anti–GFP-stained (in red) sections obtained from mice treated with HeLa cells (left) or HeLa and AD-MSC TRAIL s.c. Scale bar, 100 μm. The presence of GFP-positive cells (arrows) within tumor (T) burden confirms AD-MSC TRAIL localization.
normal tissues (16), several protocols based on rTRAIL were introduced as cancer treatment (19). However, a suboptimal half-life in plasma reduces its possible therapeutic effects. Bypassing rTRAIL limitation, agonistic anti-TRAIL-DR antibodies were generated (17). They induce a stronger antitumor effect than rTRAIL after binding to specific DR and overcoming the action of decoy receptors (49). Thought apparently favorable, this property also implies that normal cells are no longer safeguarded by apoptosis-inhibitory mechanisms and become more sensitive to apoptosis. Moreover, anti-TRAIL-DR antibodies have a longer biological half-life than rTRAIL (21 days versus 60 minutes), potentially increasing the risk of side effects (16, 50).

To overcome the mentioned limitations of rTRAIL and anti-TRAIL-DR antibodies, other researchers very recently reported the use of GM human progenitors as tools to specifically deliver TRAIL (45–47, 51, 52). Although these preliminary approaches provided relevant insights about strategies to deliver TRAIL, they mainly rely on adenoviruses that retain limits due to a subefficient gene modification of MSC lacking CAR receptors (45, 53) and to a transient transgene expression (51). Taking into account these limits, we generated a stable retrovirally transduced population of AD-MSC able to constantly produce TRAIL up to 20 passages (data not shown) without signs of abnormal cellular behavior either in vitro or in vivo, as reported by others using adipose and marrow MSC (2, 54).

Most importantly, our data indicate that AD-MSC TRAIL exert a robust cytotoxic effect on target cell lines and, in particular, on HeLa cells. It has been reported that HeLa cells are sensitive to rTRAIL (55); however, this is the first time to our knowledge that a cell therapy approach based on TRAIL has been successfully introduced to induce cervical carcinoma apoptosis. We further validate these data testing other cell lines representative of deathly tumors, such as pancreatic and colon cancers. Moreover, in association with a TRAIL-sensitizing agent such as bortezomib, we originally induce apoptosis in a TRAIL-resistant breast cancer cell line, indicating that bortezomib could be combined with a cell-based TRAIL delivery to successfully target TRAIL-resistant cancers.

In addition, in vitro data dealing with cytotoxicity induced by AD-MSC TRAIL against primary cancer cells indicate how a cell-based TRAIL delivery may be effective for the treatment of incurable cancers. Beside this approach has been tested on a single sample, due to technical issues on primary tumor cell isolation, we retain it may represent a valid proof of concept that certainly merits further validation.

Having shown that GM AD-MSC can simultaneously express TRAIL either as MB protein or as soluble ligand, we show that TRAIL effect is mainly based on a cell-to-cell contact. Even if soluble TRAIL has been detected in the culture media, its low concentration and/or the lack of stable TRAIL trimerization may not be sufficient to trigger cell death, as previously reported (56). Moreover, the TRAIL receptor profile on HeLa, with a predominant TRAIL-R2/DR5 expression, further confirms that cell-to-cell contact is preferred in our system because this receptor is preferentially activated by MB TRAIL (51, 56).

Confirming the in vitro results, two different in vivo delivery models indicate that AD-MSC TRAIL inhibit cancer growth. When directly injected into the tumor burden, AD-MSC TRAIL are integrated within its stroma and generate a cytotoxic cell bundle around tumors, suggesting a non-random persistence. Similarly, when i.v. injected in a s.c. established tumor, AD-MSC TRAIL maintain an antiproliferative effect. Although with a less prominent effect than s.c. injected AD-MSC TRAIL, the systemic cell infusion significantly reduces tumors. These findings originally suggest that i.v. infused AD-MSC can circulate over the lung vascular bed and migrate into s.c. growing tumors, supporting the concept that even AD-MSCs, similarly to other progenitors, home into tumors (1, 13, 45).

We also have to report a more prominent tumor growth in mice treated with AD-MSC GFP in comparison with HeLa alone, similarly to what has been described for WT BM-MSC (57). Interestingly, this trend decreases over time and, in late time points, seems reversible. The reason behind this phenomenon is under investigation; it may be that the ratios between AD-MSC and tumor cells can initially be in favor of a proliferative burst; however, when cancer cells become prevalent, the amount of AD-MSC may not be adequate to feed tumor. Nevertheless, the antiproliferative effect exerted by TRAIL-producing AD-MSC is able not only to counterbalance the tumor-supportive capacity of AD-MSC but also to determine a powerful inhibitory effect.

Because rTRAIL has been previously associated with liver toxicity (24), we wanted to investigate whether tumor treatment with AD-MSC TRAIL may be similarly related with this side effect. In TRAIL-treated mice, liver enzyme levels are normal and liver histology does not provide evidence of abnormal features.

Conclusively, our data indicate that a cell therapy approach with stably genetically modified AD-MSC delivering TRAIL alone or in combination with sensitizing agents is opening novel therapeutic opportunities for still incurable cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Ministero Italiano Istruzione Università e Ricerca grants PRIN 2006 and PRIN 2008 (M. Dominici), OTKA-T046665, ETT 576/2006, and OMFB-00257/ KK; National Office for Research and Technology Hungary (I. Petak); NIH grant R01 HL077643 (E.M. Horwitz); Regione Emilia Romagna (P. Paolucci, P. Conte, and M. Dominici); Associazione per il Sostegno dell’Eematologia e dell’Oncoologia Pediatrica (P. Paolucci); and Fondazione Cassa di Risparmio di Modena (M. Dominici).

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Received 05/21/2009; revised 02/23/2010; accepted 02/24/2010; published OnlineFirst 04/13/2010.


Adipose-Derived Mesenchymal Stem Cells as Stable Source of Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Delivery for Cancer Therapy

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Cancer Res 2010;70:3718-3729. Published OnlineFirst April 13, 2010.

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doi:10.1158/0008-5472.CAN-09-1865

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