Genotoxic damage of human adipose-tissue derived mesenchymal stem cells triggers their terminal differentiation

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Human adipose tissue-derived mesenchymal (stromal) stem cells (AT-MSCs) and genetically modified to express cytosine deaminase:uracil phosphoribosyltransferase (CDy-AT-MSCs) were treated with hydrogen peroxide in order to induce DNA damage and subsequently evaluate their genetic stability by single cell gel electrophoresis. Both cells types (parental and transgene modified) did not differ in the sensitivity to DNA breaks induction. Potential tumorigenicity of AT-MSCs and CDy-AT-MSCs was tested by subcutaneous inoculation of cell suspension into flank of immunocompromised mice. Dose of 15x10^6 cells was not found to be tumorigenic in given experimental setup. AT-MSCs, CDy-AT-MSCs and MSCs isolated from human lipoma were treated with chemical carcinogen 4-nitroquinoline-1-oxide (4NQO) in attempts to transform them. Surviving cells after genotoxic stress were not transformed but underwent replicative senescence. Irreparable DNA damage caused triggered adipogenic terminal differentiation, rather than apoptosis induction in all kinds of cells tested.

Key words: human adipose tissue-derived mesenchymal stem cells; genotoxic damage, single cell gel electrohoresis; chemical carcinogen 4NQO, terminal differentiation

Human adipose tissue-derived mesenchymal (stromal) stem cells (AT-MSCs) are multipotent adult stem cells derived usually from lipoaspirate. Similarly as bone marrow derived mesenchymal stem cells (MSC), AT-MSCs have the ability to migrate to sites of injury to fulfill their role in repair of the damage tissues [1]. MSCs and AT-MSCs can be isolated by their adherence to plastic tissue culture plates [2]. The yield of MSCs from adipose tissue is higher in comparison to the bone marrow [1, 3, 4]. The easy and repeatable access to subcutaneous adipose tissue and the simple isolation procedures provides a clear advantage over other sources [4]. Therefore AT-MSCs have potential therapeutic use as autologous and allogeneic products for tissue engineering.

Mesenchymal stem cells both MSCs and AT-MSCs possess unique ability to selectively migrate to tumors, metastases and contribute to the formation of tumor-associated stroma. This property precludes them to become vehicles for stem cell based targeted cancer gene therapy [5, 6].

Previously we have prepared yeast fusion cytosine deaminase:uracil phosphoribosyltransferase expressing human adipose tissue derived mesenchymal stem cells (CDy-AT-MSCs) by retrovirus transduction. CDy-AT-MSCs exerted their anti-tumor potential on human colon cancer cells, human malignant melanoma cell line, and human metastatic prostate cells in the presence of prodrug 5-fluorocytosine (5-FC) [3, 7, 8]. CDy-AT-MSCs in combination with 5-FC augmented significant bystander effect and selective cytotoxicity on target tumor cells in co-culture in vitro. Moreover, strong inhibition of subcutaneous tumor growth was achieved by systemically administered CDy-AT-MSCs on xenotransplanted human tumors grown on nude mice upon 5-FC treatment.

Therefore human mesenchymal stem cells derived from adipose tissue are not only useful cells in regenerative medicine, but when modified by prodrug converting gene may also serve as attractive delivery vehicles for stem cell-based targeted cancer gene therapy. Genetic stability of AT-MSCs is an important attribute to assess, when they are intended to be used in regenerative and cancer gene therapy clinical studies.

The aim of the present study was to evaluate genetic stability, of AT-MSCs and CDy-AT-MSCs, their sensitivity to transformation induced by chemical carcinogen and potential tumorigenicity. Here we demonstrate that irreparable DNA damage caused by chemical carcinogen triggered adipose...
derived mesenchymal (stromal) cells to adipogenic, terminal differentiation.

Materials and methods

Chemicals. Ethidium bromide, 4-nitroquinoline-1-oxide (4NQO) were purchased from Sigma-Aldrich Co. (St. Louis, MO). 4NQO was prepared as 5 mmol/L stock solutions in 100% dimethylsulfoxide (DMSO). Aliquots were diluted with complete medium just before they were applied to culture cells.

Mesenchymal stem cells isolation from human adipose tissue, culture, and retrovirus transduction. AT-MSC cells were isolated and cultivated as we described previously [7]. Lipoaspirates for their isolation were obtained from healthy persons undergoing elective lipospiration, who provided an informed consent. Cells were plated in low glucose (1.0 mg/L) DMEM supplemented with 5% MSC-stimulatory supplement (StemCell Technologies), 5% of human platelet extract and antibiotic-antimycotic mixture. AT-MSCs were cultured at 37°C in a humidified atmosphere containing 5% carbon dioxide with medium changes twice a week. Cells were plated at a density of 3x10^3 to 5x10^3 nucleated cells/cm^2. Adherent cells were split upon reaching confluence and AT-MSCs were used for the experiments up to passage 5.

To prepare AT-MSCs expressing cytosine deaminase (CDy-AT-MSCs), subconfluent cultures of AT-MSCs were transduced thrice in 3 consecutive days with virus-containing medium from GP+envAM12/pST2 cells supplemented with 100 μg/mL protamine sulphate. Transduced cells were selected by cultivation in medium supplemented with 0.5 mg/ml of G418.

Experiments in vivo. Six- to 8-week-old athymic nude mice (Balb/c-nu/nu) were used in accordance with institutional guidelines under the approved protocols. The potential tumorigenicity of AT-MSCs and CDy-AT-MSCs was tested by subcutaneous inoculation of cell suspension of 15x10^6 AT-MSCs or CDy-AT-MSCs in 0.1 ml PBS into flank of each nude mouse (n = 2 in each treatment group). Animals were inspected for tumor growth during period of time two months. The palpable cell inoculum persisted on the site of injection several days and slowly disappeared during first two weeks. By day 60 the experiments were ended and animals were sacrificed and inspected for tumor presence. No tumors were found either on the site of inoculation nor elsewhere during the autopsy. Therefore the genetically modified mesenchymal cells did not differ from parental cells in the sensitivity to DNA breaks induction.

Treatment of AT-MSCs, CDy-AT-MSCs, and MSCs from lipoma with 4NQO.

Cells were exposed to single dose of 10 μM concentration of 4NQO. Survived fraction of cells (about ten percent) was further cultivated in growth medium and when reached confluence transferred by trypsin treatment. The cultivation continued for several months. During this period of time signs of senescence appeared, intervals of cell transfer was longer, the number of passages never reach more than 10 before the terminal differentiation appeared.

Statistics. Statistical comparisons of mean tail DNA values in hydrogen peroxide treatment experiments were performed using the Student’s t-test.

Results

Induction of single stranded DNA breaks. Treatment of AT-MSCs and CDy-AT-MSCs with hydrogen peroxide resulted in an increase of DNA breaks in both parental AT-MSCs and transgene-modified CDy-AT-MSCs when concentration of hydrogen peroxide reached 100 μM. Higher hydrogen peroxide concentration did not further increase the number of DNA breaks (Fig. 1.). Percentage of tail DNA was comparable at each hydrogen peroxide concentration tested in both kinds of cells. Therefore the genetically modified mesenchymal cells did not differ from parental cells in the sensitivity to DNA breaks induction.

Tumorigenicity of AT-MSCs and CDy-AT-MSCs testing. Inoculums of cell suspension (15x10^6 of AT-MSCs or CDy-AT-MSCs) in 0.1 ml PBS was injected subcutaneously into the flank of nude mouse (n = 2 in each treatment group). Animals were inspected for tumor growth during period of time two months. The palpable cell inoculum persisted on the site of injection several days and slowly disappeared during first two weeks. By day 60 the experiments were ended and animals were sacrificed and inspected for tumor presence. No tumors were found either on the site of inoculation nor elsewhere during the autopsy. Therefore the AT-MSCs and CDy-AT-MSCs were found not to be tumorigenic in given experimental setup.

Cytotoxicity of 4NQO to AT-MSC and CDy-AT-MSC. Mesenchymal stem cells are known for their higher natural resistance to toxic agents. In order to elucidate the sensitivity to toxic agents...
of AT-MSCs, CDy-AT-MSCs and MSCs isolated from lipoma, potent chemical carcinogen and mutagen 4-nitroquinoline-1-oxide (4NQO) was chosen for cell treatment. Concentrations higher than 60 μM of 4NQO were found to be toxic to destroy cells completely during 4 days of cultivation. At least 10 percent of cells survived treatment with 10 μM concentration of 4NQO for 4 days. Recovered 4NQO-treated cells were further cultivated and split upon reaching confluence. Cell proliferation declined with passages kept in culture. Cells became more light-refractory, the cell morphology changed to cells more prolonged (Fig. 2 B). Typical signs of aging-related phenotype appeared (Fig. 2 C). Finally, the cells appeared to form drops of fat, signs of mature differentiation to adipocytes in all cells (Fig. 2 D). Accumulation of irreparable DNA damage in adipose tissue derived mesenchymal stem cells apparently triggered their terminal differentiation. Terminal adipogenic differentiation after genotoxic stress caused by 4NGO was noticed in AT-MSCs, in cytosine deaminase transduced CDy-ATMSCs and also in MSCs isolated from human lipomas.

Discussion

Several properties of human mesenchymal stem cells derived from adipose tissue designate them attractive vehicles for cell therapies. They are able to differentiate along a variety of lineage pathways, including bone, cartilage, adipose, neuronal-like, and muscle in vitro [see 1 for a review]. They are nonimmunogenic upon transplantation into allogeneic host [4, 13, 14]. The cells exhibit a low immunogenic profile as shown by negative expression of MHC class II molecules and absence of costimulatory molecule expression [15, 16, 17]. Furthermore it was found that they are immunosuppressive [18, 19] as it was demonstrated by their ability to control graft-versus-host disease (GvHD) in humans [20].

Moreover AT-MSCs possess unique ability to selectively migrate to tumors and metastases. When AT-MSCs are modified by prodrug converting gene they are attractive delivery vehicles for anti-tumor agents such as IFNβ to tumors [21] and for stem cell-based targeted cancer gene therapy [3, 7, 8, 22]. Taken in account nonimmunogenic properties of MSCs, AT-MSCs may have potential therapeutic use as allogeneic products for tissue engineering and for gene therapy upon genetic modifications as well. For all these reasons, to assess genetic stability of these cells is rather important, when they are intended to be used in clinical studies.

There were several contradictive reports in literature with regards to involvement of adult MSCs in tumor formation [23]. Increasing number of reports indicated that MSCs are recruited in large numbers to the stroma of developing tumors [21, 24], or they could act by enhancing the motility, invasion and metastasis ability of adjacent cancer cells [25].

Experiments with human xenografts on nude mice have shown that systemically administrated MSCs possess intrinsic preferential migratory ability towards breast carcinoma cells, lung metastasis of melanoma cells, intracranial glioma and colon cancer cells [26, 27]. Moreover exogenously administered MSCs could form a significant proportion of tumor mass [27]. It was demonstrated that bone marrow derived MSCs may also be involved in cancer metastasis forming pre-metastatic niche [28]. This finding supports the idea that therapeutically engineered MSCs may be used to treat metastases. Therefore the introduction of a transgene into autologous stem cells possesses an attractive cell based delivery strategy [8, 29]. On the contrary, other studies observed that MSCs may inhibit tumor growth in animal models [30, 31] and posses antitumorigenic effects on a model of Kaposi’s sarcoma [32].

Few contradictory reports appeared about spontaneous transformation of cultured bone marrow derived murine MSCs [32, 33, 34, 35] and human MSCs [36, 37]. It was...
reported that murine but not human mesenchymal stem cells generated osteosarcoma-like lesions in the lung [38]. If murine MSCs were implanted subcutaneously together with porous ceramic, host-derived sarcomas developed. Sarcomas developed only in syngenic and immunodeficient recipients, but not in allogenic hosts or when the cells were inoculated as suspension [39].

In our experiments we did not observe any tumor formation when a high dose of AT-MSCs, or CDy-AT-MSCs was inoculated subcutaneously as cell suspension in nude mice. However, when these cells are co-injected with tumor cells, they can support the growth of engrafts of human melanoma xenografts, but not human glioma cells on nude mice, due to their production of various growth factors in a paracrine fashion [40].

Mesenchymal stem cells as cells of vital importance for organism are equipped with efficient DNA repair system as well as high natural resistance to toxic agents [41]. It was therefore not surprising that DNA damage in CDy-AT-MSCs was not influenced by the transduction with cytosine deaminase as documented by our results obtained with single-cell gel electrophoresis, which represents a sensitive method for measuring DNA damage.

In order to find whether adipose tissue derived mesenchymal stem cells can be transformed in vitro by chemical carcinogen, we have chosen water-soluble highly effective carcinogen 4-nitroquinoline-1-oxide (4NQO). 4NQO is metabolized into 4-acetoxyaminoquinoline-1-oxide (Ac-4HAQO), which can form covalent adduct to deoxyguanine and deoxyadenine in DNA. The carcinogenic activity of 4NQO consist in DNA damage production and formation of irreversible topoisomerase I cleavage complexes (Top1cc) inducing recombinations. Top1cc produced by 4NQO accumulate progressively after 4NQO addition and persist to following 4NQO removal [42]. In addition 4NQO also produces oxidative damage and DNA single-stranded breaks [43]. Results of our study indicate that replicative senescence of AT-MSCs as a consequence of cumulating DNA damage is a continuous process including far reaching alterations in phenotype like the terminal differentiation. Similar observation with human

Figure 2. Adipogenic terminal differentiation of AT-MSC after accumulation of DNA damage caused by 4NQO. A – untreated control AT-MSCs; B – AT-MSCs exposed to 4NQO after serial passages became bigger; C – Senescence of damaged AT-MSCs progressed during serial passages, cells being more light refractory and sparse; D – All 4NQO-treated AT-MSCs differentiated to adipocytes. Cells were stained with Oil Red-O. Magnification 100 fold.
bone marrow derived MSC together with detail analysis on molecular level was reported [44, 45]. The DNA damage, a prime suspect in stem cell aging, causes graying and loss of melanocyte stem cells by inducing premature differentiation, without inducing apoptosis or senescence as it was reported very recently [46]. The irreparable DNA damage, as caused by ionizing radiation, abrogated renewal of MSCs in mice. Surprisingly, the DNA-damage response triggers MSC differentiation into mature melanocytes in the niche, rather than inducing their apoptosis or senescence [46].

Several other reports support the conclusion that accumulation of DNA damage may cause somatic stem cell depletion [47, 48]. Typical sign of aging in mammals is hair graying caused by the incomplete maintenance of melanocyte stem cells with age [47]. The resulting MSC depletion leads to irreversible hair graying. Deficiency of Ataxia-telangiectasia mutated (ATM), a central transducer kinase of the DNA-damage response, sensitizes MSCs to ectopic differentiation, demonstrating that the kinase protects MSCs from their premature differentiation by functioning as a “stemness checkpoint” to maintain the stem cell quality and quantity [49].

We observed that genotoxic damage of human adipose tissue derived mesenchymal stem cells caused by chemical carcinogen 4NQO is not leading to cell transformation but by inducing their apoptosis or senescence [46]. The elimination of DNA damaged stem cells is generally provided by terminal differentiation rather than apoptosis remains to be proven.

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