Adipose stem cells as a novel feeder layer in regenerative medicine: effects of irradiation on cell growth

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INTRODUCTION

Feeder cell layers usually consist of adherent growth-arrested but viable and bioactive cells that have been incapacitated, for example, by irradiation; these cells are used as a substratum on which other cells are grown in a co-culture system. Many cell types are dependent on physical contact with the feeder layer for survival and expansion; moreover feeder cells provide an intact functional extracellular matrix, matrix-associated factors, cytokines, soluble or membrane-bound growth factors and receptors. Usually, cells like epidermal keratinocytes (Rheinwald J.G. 1975), corneal epithelial cells (Pellegrini G. 1997), urothelial cells (Schaefer B.M. 1998), embryonic stem cells (Thomson J.A. 1998) and hematopoietic progenitor cells (Ye Z.Q. 1994) are plated with the feeder cells, which have the capacity to support cell growth.

In view of the fact that the most common feeder cells are Murine Embryonic Fibroblasts well-known as 3T3 (Rheinwald J.G. 1975) and that recently cells cultured on a 3T3 feeder layer, and transferred to human, have been considered as a xenograft by the US FDA (Sugiyama H. 2008), it is imperative that novel human feeder layers must be developed. Furthermore, animal feeder cell layers can incorporate non human sialic acids, leading to a high immunogenic risk (Martin M. 2005); again, 3T3 can be a carrier of xenoinfection (Takeuchi T. 2008). In 2008, Sugiyama et al. proposed the employment of adipose stem cells (ASC) as a feeder layer. These cells can be obtained by minimal invasive methods from adipose tissue, in large quantities; moreover these cells grow easily in standard tissue culture conditions and can be expanded more rapidly than bone marrow-stem cells, maintaining their phenotype and pluripotency (Zhu Y. 2008), as well as secreting several collagens, fibronectin and growth factors (Kim W.S. 2007, Gimble J.M. 2007).

The aim of this work is to characterize the *in vitro* behaviour of adipose stem cells as a good human alternative source of feeder cell layers: growth patterns were determined for irradiated and non-irradiated cells.

MATERIALS AND METHODS

Cell culture

Human subcutaneous adipose tissue samples were obtained during abdominoplasty; healthy female donors provided written informed consent. The harvested tissue was processed using a modified procedure by Zuk et al. (2002). Briefly, the samples were digested with 0.075% collagenase type II (Sigma–Aldrich, St. Louis, MO) for 45 min at 37° C, centrifuged at 300 g for 10 min and the supernatant was discarded. The pellet was rinsed three-time in phosphate buffered saline (PBS), centrifuged and resuspended in PBS. The recovered cell fraction was counted using trypan blue exclusion technique, seeded in culture dishes at a density of 100.000 cells/dish and cultured in Dulbecco's modified Eagle containing 10% fetal bovine serum, 100 U/ml of penicillin, 100 mg/ml of streptomycin and 25 mg/ml amphotericin B. At the same time, adipose stem cells were irradiated (60 Gy X-rays) as reported for the 3T3 cells (Rheinwald J.G. 1975), and seeded onto 20 culture

dishes at the same conditions. Fresh medium was added after 4, 8, 14 and 18 days of culture, while at day 10 all medium was removed and replaced with a fresh medium.

Measurement of cell mortality and doubling time

The irradiated and non-irradiated cell growth kinetics were monitored for 20 days: at intervals of 48 hours, two dishes (one for each condition) were trypsinized to determine the number of buoyant, viable and dead cells. Cells were counted 2 times per sample under the microscope using the trypan blue exclusion technique. Doubling time (Td) (hours) of cells was calculated in according to the formula: $Td = t / \log_2(N_2/N_1)$, where N_1 stands for the cell count at time t (days) and N_2 stands for the cell count at time $t+\Delta t$. All experiments were performed in duplicate and cells were not synchronized.

Statistical analysis

Results for the number of total and live cells for each treatment (irradiated and non-irradiated cells) are reported as the mean of Log_2 –transformed data as a function of culture time. Percentages of live cells for each treatment as a function of time are reported as mean value ± standard deviation. The effects of time and treatment for each variable in each group are analyzed by a two-way ANOVA, considering treatment and time as fixed effects. Statistical significance was set at p=0.05. All analyses were performed with JMP 7 for Windows (SAS Institute).

RESULTS AND DISCUSSION

Adipose stem cells have a high proliferative capacity: as shown in Figure 1 non-irradiated cells have an exponential growth phase in about 6 days, a stationary phase for 10-12 days and a phase of decline over 14 days. X-ray irradiation significantly alters the growth ability of adipose stem cells; in fact irradiated cells have an exponential growth phase in about 4 days, followed by a phase of marked decline from 16 to 20 days. This decline is clearly shown for live cells (Figure 2). ANOVA analysis shows a significant effect of time and treatment in each group ($p<0.01$) both for total and live cells.

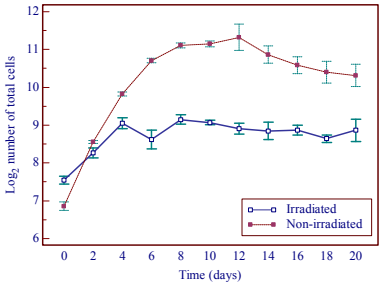

Figure 1: Growth kinetics of irradiated and non-irradiated total cells.

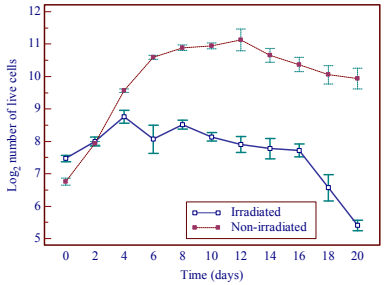

Figure 2: Growth kinetics of irradiated and non-irradiated live cells.

Non-irradiated and irradiated cells growth kinetics are also reported as mean values ± st.dev of N2/N1 and Td (table 1). Non-irradiated cells present a doubling time of about 47 hours in the exponential phase (6 days); irradiated cells have a doubling time of about 75 hours in 4 days and, over this time, cells lose their proliferative capacity. In fact X-rays cause considerable DNA damage and induce cells to apoptosis (Haimovitz-Friedman A. 1994; Kadhim M.A. 1995). Irradiated cells

repair damages and complete the cell cycle, but time after time they induce programmed cell death. Probably, cell death is under-estimated because Trypan Blue stain is able to penetrate cell membrane only when apoptosis is advanced.

Time	Non-irradiated cells		Irradiated cells	
Days	N_2/N_1	Td	N_2/N_1	Td
2	2.28±0.25	34.65±4.74	1.44±0.06	77.64±9.90
4	3.08±0.17	33.04±1.57	1.71±0.26	75.07±22.87
6	2.05±0.10	46.61±3.47	0.63±0.12	-82.83±48.38
8	1.23±0.11	194.91±102.78	1.42±0.46	-349.2±1010.03

Table 1: Growth kinetics (mean values ± st.dev) of non-irradiated and irradiated cells: N_1 cell count at time t and N_2 cell count at t+Δt; Td: doubling time (hours).

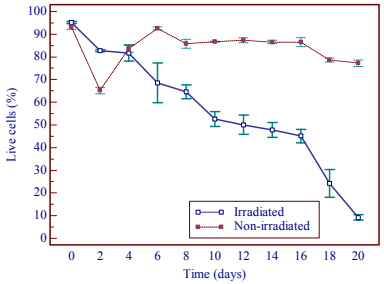

Figure 3: Percentage of irradiated and non-irradiated live cells over time.

In Figure 3, growth kinetic is reported as percentage of live cells in function of time; in irradiated cells, death rate is readily observable. Non-irradiated cells present a nadir at day 2, probably associated with the trypsinization process.
The effect of irradiation can be directly observed in microscopical images: cytoplasmic extensions and round-shaped cytoplasmic fragments are appreciable in irradiated cells (Figure 4), whereas high concentration of adherent viable cells characterizes non-irradiated cultures (Figure 5).

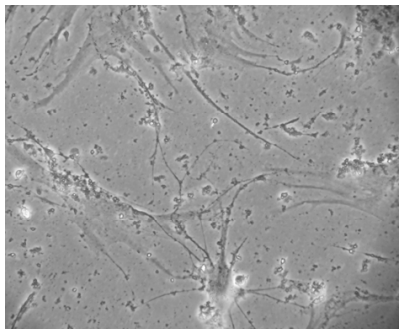

Figure 4: Irradiated cells. 13 days of culture. Magnification 40X.

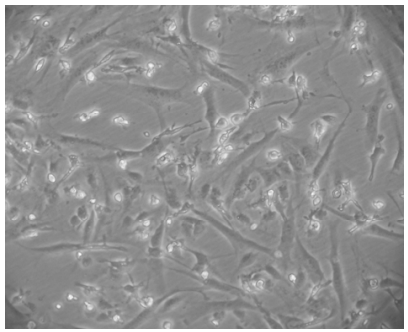

Figure 5: Non-irradiated cells. 13 days of culture. Magnification 40X.

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CONCLUSIONS

The results indicate that irradiated cells develop a death grade in six days of culture and this phenomenon becomes highly evident after 16 days. Adipose stem cells represent human alternative source of feeder layers, and could be employed in tissue engineering to increase cell adhesion and for the *in vitro* proliferation of cells such as chondrocytes, keratinocytes and osteoblasts, which are commonly used in cell therapy. Moreover, the autologous irradiated ASC in regenerative medicine could be safer than non-irradiated cells: they could *in vivo* promote the healing of damaged tissues by paracrine mechanisms avoiding the risks linked to an excessive ASC proliferation.

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