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Basic Science

A COMPARISON OF AMNIOTIC, UMBILICAL CORD, ADIPOSE AND BONE MARROW MESENCHYMAL STROMAL CELLS ON SOFT AGAR ASSAY

Wong HS* Australian Women's Ultrasound Centre, Hong Kong *Corresponding Author

ABSTRACT The tumorigenicity of amniotic, umbilical cord, adipose and bone marrow mesenchymal stromal cells were assessed in-vitro with soft agar assay using a Z-stack volume on microscopy. HeLa cells were used as positive controls. Colony formation was observed with all 5 types of cells. However, the total area of colonies and the percentage area of the colonies of the 4 types of mesenchymal stromal cells were statistically significantly less when compared with HeLa cells. No statistical significant difference was noted when amniotic, umbilical cord and adipose mesenchymal stromal cells were compared to bone marrow cells.

KEYWORDS : Mesenchymal stem cell, amnion, umbilical cord, adipose, bone marrow, soft agar assay

Introduction

Stem cells are by definition pluripotent and could differentiate into cells of 3 germ layers forming teratomas in-vivo. Tumorigenicity is a major consideration when stem cells are used therapeutically. Testing for tumorigenicity is recommended during characterization of cell line by WHO, either in-vivo or in-vitro. While in-vivo tests are the standard for assessing tumorigenicity[1], they are expensive and time consuming.[2] Although in-vitro tests (e.g. soft agar colony formation assay) are not perfect, they can be used as alternatives under most circumstances.[1,2] In this paper, the tumorigenicity of four types of mesenchymal stem cells (MSCs), namely amniotic, umbilical cord, adipose and bone marrow, will be assessed and compared with HeLa cells using an in-intro method, soft agar colony formation assay.

Soft agar colony formation assay has been recognized as a conventional in-vitro method to monitor anchorage-independent growth for the detection of malignant transformation of cells.[3,4] The growth of normal cells require the interactions between the cells and the intercellular matrix whereas transformed cells (e.g. cancer cells, pluripotent cells) do not. Essentially, the assay involves the layering of agar-cell mixture on top of another agar layer which prevents cellular adhesion onto the culture plate. The formation of colonies in agar with time is then observed as evidence of cell transformation.[4] The method used here is based on the protocol published by Borowicz S. et.al. (2014).[4]

Comparison of tumorigenicity between different cell lines could be done semi-quantitatively by the macroscopic or microscopic appearance of the agar plate for the stained colonies or quantitatively by counting the colonies with or without the use of computer software.[5] We would like to report a novel method of analysis by counting the colonies within the whole thickness of the agar, instead of a single plane on light microscopy, and compare quantitatively the tumorigenicity of amniotic, umbilical cord, adipose to bone marrow MSCs and to HeLa cells.

Objectives

To compare the tumorigenicity of amniotic, umbilical cord, adipose to bone marrow MSCs and to HeLa cells using soft agar assay

Method and materials:

All the procedures were performed in the Biosafety Cabinet. 2x and 1x culture media were prepared with the addition of antibiotics (1% penicillin/streptomycin) and filtered to sterilize. 1% and 0.6% Noble Agar were prepared and autoclaved. 1% Agar solution and 2x DMEM (Thermo Fisher Scientific, U.S.A.) were warmed and mixed in equal volumes. 1.5 millilitre (ml) of the mixture was used to plate the bottom of 3 wells of a 6 wells plate. When this bottom layer had set, the respective mesenchymal stem cells were added into complete Stempro culture medium supplemented with 2.5% platelet lysate and 1% penicillin/streptomycin to give a concentration of 6667 cells per ml. An equal volume of MSCs in supplemented complete Stempro medium mixture and 0.6% Agar solution were mixed and 1.5ml of the mixture was layered on top of each well (giving a final concentration of 5000 cells per well). The plate was cooled to allow the agar to set and then incubated at 37°C in 5% CO_2 in humidified incubator for 21 days. 100ul of supplemented complete Stempro medium was added to each well twice a week. At the end of the incubation period, the cells were stained in the plate with 0.1% Crystal violet solution for 30 minutes, and then washed with copious amount of phosphate buffered saline (PBS). The plate was viewed under inverted microscope (Axiovert 25, Zeiss, Germany). A Z-stack image corresponding to 3000 (Width) x 2150 (Depth) x 2000 (Height) µm was taken at the centre of the 3 wells from the top to the bottom of the cellular agar layer at 20µm interval with 4X objective (Evos FL Auto, Thermo Fisher Scientific, U.S.A.). The colonies were then analyzed with Image J software[5] The background of fine cellular deposits were filtered with the software until only colonies with irregular constellation appearance remained (Fig. 1) and these colonies were counted. for the total number of colonies, the total area of colonies and the percentage of area being occupied by colonies The procedure was performed with amniotic, umbilical cord, adipose, bone marrow MSCs and HeLa cells respectively and the results were compared (Table 1&2, Fig. 2-5).

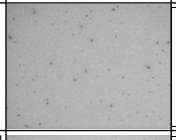
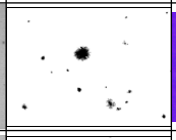
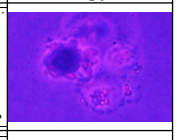
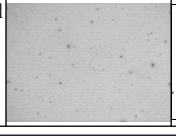
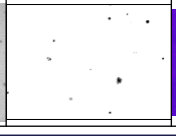
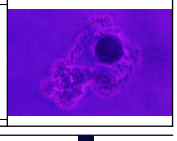
For each of the 5 cell types, a single plane was selected from the middle of each of the 3 Z-stack volumes, and the total number of colonies, the total area of colonies and the percentage of area being occupied by colonies were assessed with Image-J as described above. The mean from the 3 single planes was calculated and compared with that of the Z-stack volumes (Fig. 6-9). The correlation between the total number of colonies, the total area of colonies and the percentage of area on the single planes and the Z-stack volumes for all 5 types of cells was analyzed with paired-samples t-test and shown in Table 3.

Statistical analysis was performed with Statistical Package for the Social Sciences Version 21.0 (SPSS Inc., Chicago, IL). A 2-sided probability value of <0.05 was taken as statistical significant.

For box plot, the box indicates the upper and lower quartiles, and the interior of the box plot the inner-quartile range. The area between the upper and lower quartiles comprises of 50% of the distribution. The whiskers refer to 1.5 multiple of the inner-quartile range. The crossbar indicates the median.

Results:

The Z-stack images, the appearance on Image-J and on light microscopy of the colonies of the 5 types of cells were shown in Fig. 1. Colony formation was observed with all 5 types of cells. The appearance of individual colony of the respective MSCs and HeLa cells was illustrated in Fig. 1 and also in a previous publication by the author.[6]

Cell type	Z-stack image of colonies	Colonies on Image J	Colonies on phase contrast light microscopy
Amniotic			
Umbilical cord			

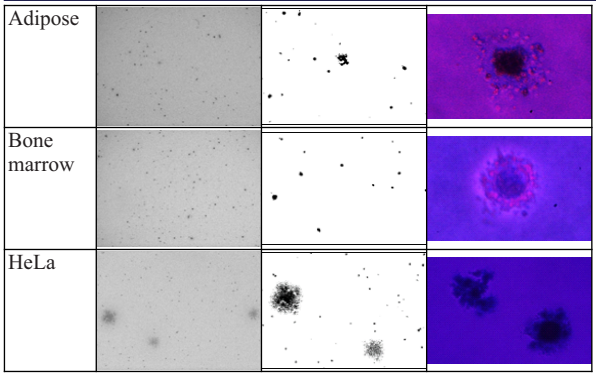


Fig. 1: The appearance of the colonies of amniotic, umbilical cord, adipose and bone marrow MSCs and HeLa cells on soft agar assay on phase contrast light microscopy and Image J program.

A section of the pictures of the colonies on image J have been selected, enlarged and shown here to illustrate the irregular constellation appearance of the colonies of the respective MSCs.

The count of colonies, the total area of colonies, the average size of colonies and the percentage area of the colonies were compared to HeLa cells on Z-stack volumes in Table 1 and to bone marrow cells in Table 2, and illustrated in Fig. 2 to 5 respectively. The total area of colonies and the percentage area of the colonies of amniotic, umbilical cord, adipose and bone marrow MSCs were statistically significantly less when compared with HeLa cells (p = 0.01, 0.005, 0.005 and 0.009 respectively). The count of colonies of the 4 types of MSCs showed a lesser trend, though not statistically significantly different from that of HeLa cells (p = 0.07, 0.06, 0.06 and 0.07 respectively) (Table 1). However, when amniotic, umbilical cord and adipose were compared with bone marrow MSCs, no statistical significant difference was observed in terms of the count, the total area, the average size and the percentage of area of colonies (Table 2).

Table 1: Comparison of the colonies of amniotic, umbilical cord, adipose and bone marrow MSCs against HeLa cells on soft agar assay

Cell type	Colonies							
	Count		Total area (units)		Average size (units)		Percentage of area on agar plate (%)	
	(mean ± S.D.)	p†	(mean ± S.D.)	p†	(mean ± S.D.)	p†	(mean ± S.D.)	p†
Amniotic	153.3 ± 44.1	0.07	3262.0 ± 1302.1	0.01*	20.9 ± 2.5	0.8	0.27 ± 0.11	0.01*
Umbilical	103.0 ± 39.3	0.06	2186.7 ± 729.5	0.005*	21.8 ± 4.1	0.7	0.18 ± 0.06	0.005 *
Adipose	96.3 ± 33.6	0.06	2313.3 ± 245.8	0.005*	25.7 ± 8.0	0.5	0.19 ± 0.02	0.005 *
Bone marrow	123.0 ± 76.4	0.07	2995.0 ± 1266.9	0.009*	26.2 ± 4.9	0.4	0.24 ± 0.10	0.009 *
HeLa	779.3 ± 444.2	1	12113.0 ± 3052.6	1	19.4 ± 10.7	1	0.99 ± 0.25	1

S.D., standard deviation; p, probability; †, independent sample t test, equal variances assumed; *, statistically significant, 2-tailed

Table 2: Comparison of colonies formation between amniotic, umbilical cord, adipose and bone marrow MSCs on soft agar assay

Cell type	Colonies							
	Count		Total area (units)		Average size (units)		Percentage of area on agar plate (%)	
	Mean ± S.D.	p†	Mean ± S.D.	p†	Mean ± S.D.	p†	Mean ± S.D.	p†
Amniotic	153.3 ± 44.1	0.6	3262.0 ± 1302.1	0.8	20.9 ± 2.5	0.2	0.27 ± 0.11 %	0.8
Umbilical cord	103.0 ± 39.3	0.7	2186.7 ± 729.5	0.4	21.8 ± 4.1	0.3	0.18 ± 0.06 %	0.4
Adipose	96.3 ± 33.6	0.6	2313.3 ± 245.8	0.4	25.7 ± 8.0	0.9	0.19 ± 0.02 %	0.4
Bone marrow	123.0 ± 76.4	1	2995.0 ± 1266.9	1	26.2 ± 4.9	1	0.24 ± 0.10 %	1

S.D., standard deviation; p, probability, 2-tailed; *, statistical significant; †, independent samples t test, equal variance assumed

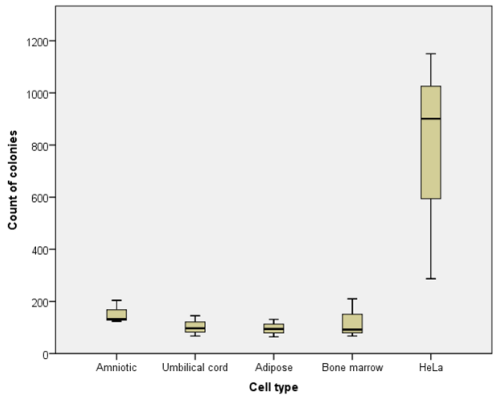


Fig. 2: The count of colonies of amniotic, umbilical cord, adipose and bone marrow MSCs as compared to HeLa cells

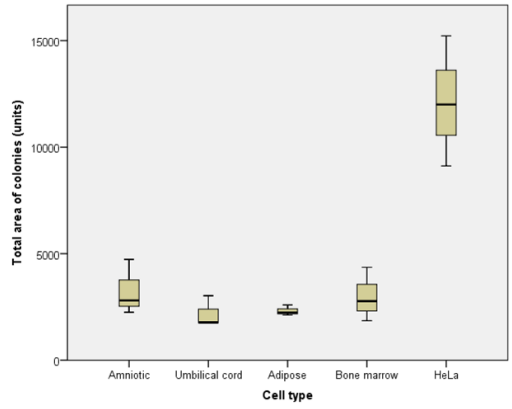


Fig. 3: Total area of soft agar being occupied by colonies of amniotic, umbilical cord, adipose and bone marrow MSCs compared to HeLa cells

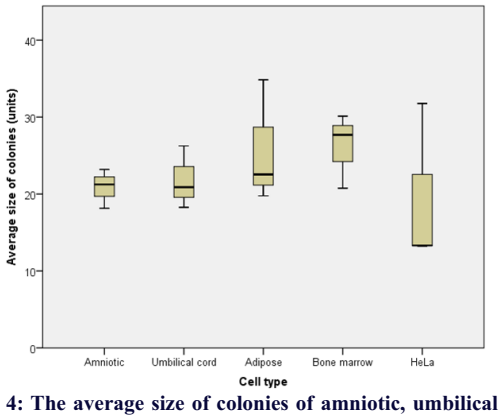


Fig. 4: The average size of colonies of amniotic, umbilical cord, adipose and bone marrow MSCs as compared to HeLa cells

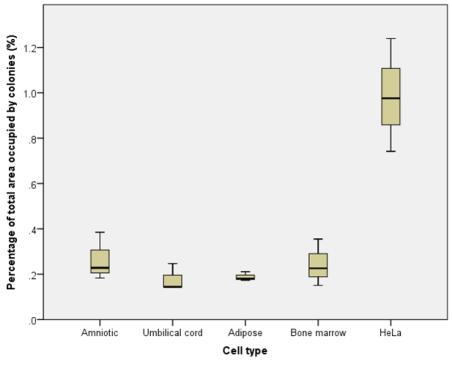


Fig. 5: The percentage of total area of soft agar being occupied by colonies of amniotic, umbilical cord, adipose and bone marrow MSCs as compared to HeLa cells

When the mean colony count, total area of colonies, average area of colonies and percentage area occupied by colonies on single planes were compared to that on Z-stack volumes for all 5 types of cells (Fig. 6-9), the paired-sample correlation only showed statistical significance for the total area of colonies and percentage area occupied by colonies (Table 3).

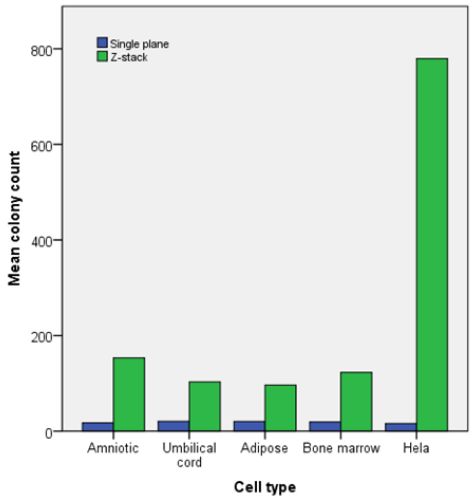


Fig. 6: A comparison of the mean colony count assessed on single planes versus Z-stack volumes for amniotic, umbilical cord, adipose, bone marrow and Hela cells.

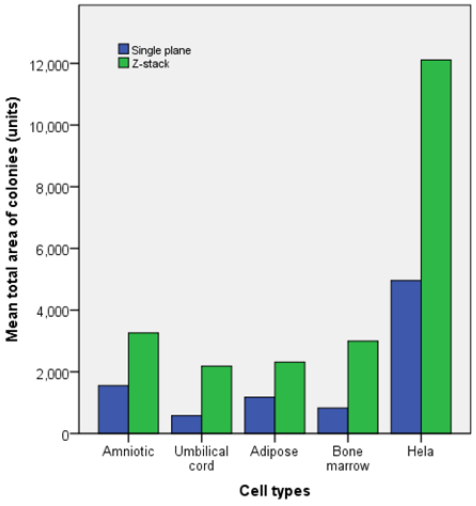


Fig. 7: A comparison of the assessed mean total area of colonies on single planes versus Z-stack volumes for amniotic, umbilical cord, adipose, bone marrow and Hela cells.

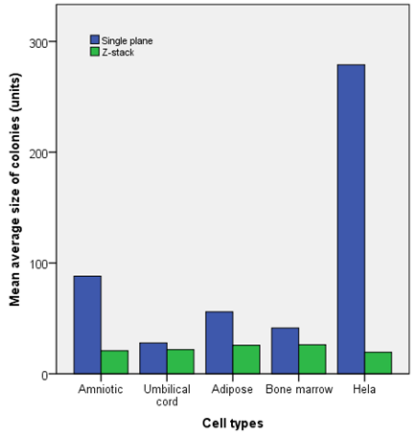


Fig. 8: A comparison of the assessed mean average areas of colonies on single planes versus Z-stack volumes for amniotic, umbilical cord, adipose, bone marrow and Hela cells.

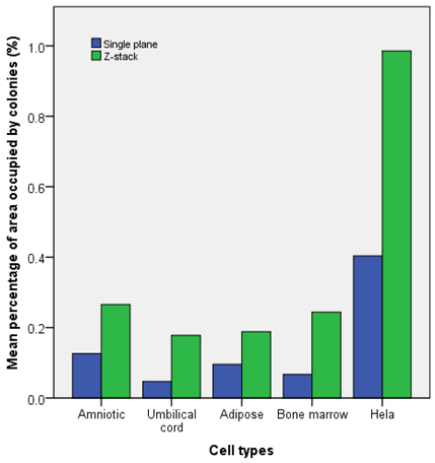


Fig 9: A comparison of the assessed mean percentage of area occupied by colonies on single planes versus Z-stack volumes for amniotic, umbilical cord, adipose, bone marrow and Hela cells.

Table 3: Correlation of the mean colony count, total area of colonies, average area of colonies and percentage area occupied by colonies on single planes to that on Z-stack volumes for amniotic, umbilical cord, adipose and bone marrow MSCs and HeLa cells.

	Paired-samples correlation		Paired-samples t-test	
	Correlation Coefficient	p†	Mean ± S.D.	p†
Mean colony count	-0.807	0.1	-232.3 ± 297.8	0.2
Total area of colonies	0.987	0.02*	-2755.5 ± 2486.9	0.07
Average size of colonies	-0.672	0.2	75.6 ± 105.4	0.2
Percentage area occupied by colonies	0.987	0.02*	-0.2 ± 0.2	0.07

Discussion

Soft agar assay has been used to assess tumorigenicity and chemosensitivity of tumour cells.[3,7,8] It has been suggested that the colonies observed arise from clonal growth of cell aggregates rather than single cells.[9] The microscopic appearance of the colonies in this study shows that the colonies demonstrate cellular outgrowths giving rise to an irregular constellation appearance (Fig. 1). With the use of computer software, single cells or small aggregates of cells in the background could be eliminated, allowing colonies to be assessed and compared quantitatively.

When the use of single planes on microscopy was compared to the Z-stack volumes for assessment of the colonies, it can be seen from Fig. 6, 7 & 9 that the mean colony count, the total area and the percentage area of colonies are larger in Z-stack volumes. This is comprehensible as more colonies will be picked up with the 3-dimensional volume rather than a single plane. However, the average size of the colonies appears to be larger on single planes than the Z-stack volumes (Table 3). This is because smaller colonies that are missed on single planes could be picked up with the Z-stack volumes. Out of the 4 parameters used, a statistically significant correlation could only be seen with the total area and percentage area of colonies between the use of single planes and Z-stack volumes (Table 3). As some of the colonies could be missed with the use of single planes, the application of 3-dimensional Z-stack volume could be a better assessment method for colony formation.

In this study, amniotic, umbilical cord, adipose and bone marrow MSCs appeared to be able to form colonies, though to a much lesser extent when compared to HeLa cells (Table 1, Fig. 2-5). However, when amniotic, umbilical cord and adipose MSCs are compared to bone marrow, no statistically significant difference could be detected in terms of the count, the total area, the average size and the percentage area of colonies (Table 2, Fig. 2-5). In a previous publication where anchorage-independent growth was not observed with umbilical cord mesenchymal stromal cells[10], the duration of growth allowed was 7-14

versus 21 days in this study. With in-vivo animal study, Fong et.al. (2007)[11] and Ilancheran et.al. (2007)[12] claimed no teratomas formation observed with umbilical cord and amniotic mesenchymal stromal cells respectively. In a clinical trial, Centeno et.al. (2010) also reported with MRI no neoplastic complications at any autologous bone marrow mesenchymal stromal cells implantation site on the treatment of orthopaedic conditions in 339 patients. Colony formation of amniotic, umbilical cord, adipose and bone marrow MSCs appear to be relatively low uniformly compared to HeLa cells in this study. Although this is consistent with the results of previous in-vivo studies, it remains It remains uncertain if the low in-vitro colony formation could be converted to low tumorigenicity potential in-vivo, and caution is to be exercised when MSCs are applied in-vivo in general.

Conclusion

Amniotic, umbilical cord, adipose and bone marrow MSCs show statistically significantly less area of colony formation than HeLa cells on soft agar assay. No statistical significance could be observed when amniotic, umbilical cord and adipose MSCs are compared to bone marrow cells. The use of Z-stack volumes instead of single planes on microscopy may allow a better assessment of colony formation.

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