Introduction

In the 1960s, Alexander Friedenstein, a Soviet researcher, discovered multipotent progenitors of conjunctive tissue in bone marrow. Cultivation of the bone marrow cells of rabbits or rodents at low density in a liquid medium containing serum revealed colonies of long, plastic-adherent cells with fibroblastic appearance [1]. These colonies came from only one cell type,
named colony-forming unit-fibroblasts (CFU-Fs). The cells were quiescent at the beginning of culture, but cultured adherent cells, sown under the renal capsule of syngeneic animals, generated fibrous tissue, bone or osseous trabecula containing hematopoietic marrow (in a proportion of approximately 1:3 for each). Study of chimeric animals showed that the fibrous tissue and bone came from the donor and the hematopoietic tissue from the receiver [1].

Mesenchymal progenitors are found in the bone marrow of many mammal species. However, their frequency and potential for cultivation differs among species. Thus, in rodents, mesenchymal progenitors of rat are easy to cultivate but not those of mouse. Only those of the Balb/C and FVB/N strains really proliferate [2]. The growth of CFU-Fs from other mouse strains is inhibited by contaminating macrophages, which are very difficult to eliminate without depleting cell numbers [3]. The presence of CFU-Fs in humans was described in 1980 [4].

Arnold Caplan, at the beginning of the 1990s, named CFU-Fs mesenchymal stem cells (MSC) and showed that they generate not only bone and medullar stroma but also cartilage, tendons, and muscle [5]. The cells are also known as marrow stromal cells, and in 2005, the International Society for Cellular Therapy clarified the acronym MSC as multipotent mesenchymal stromal cell [6], because evidence is lacking that these cells have all properties of stem cells, as defined by Potten and Loeffler [7].

**Mesenchymal Cell Sources**

Since the first description of CFU-Fs, the most studied source of mesenchymal cells is bone marrow. MSCs are present in the mononuclear fraction of medullar cells. The quantification of CFU-Fs allows for estimating the number of MSCs in bone marrow to be between 1 per $10^4$ and 1 per $10^5$ mononuclear cells. A significant factor affecting this frequency is the age of the donor. The number of CFU-Fs is higher in children than in adults: 29 per $10^6$ as compared with 3.2 per $10^6$ mononuclear cells [8]. This frequency also depends on the technique of aspiration: the concentration of CFU-Fs per milliliter decreases with increased volume of aspirated marrow for each puncture because of blood dilution [9]. Bone marrow is commonly aspirated from the sternum or iliac crest. It can also be obtained from vertebrae [10]. The concentration of CFU-Fs is 70% higher for this last site. Some pathological conditions or treatments have an effect on the number of MSCs in bone marrow. Indeed, the frequency of CFU-Fs is abnormal in patients with aseptic osteonecrosis of the femoral head related to corticosteroid therapy [11]; no MSCs are found in the heart of the necroses, and their frequency is decreased in the femoral head and iliac bone although their potential for differentiation does not seem to be compromised [12].

MSCs can also be obtained directly from trabecular bone. Thus, the osseous fragments obtained at the time of total prosthesis of the hip are a source of MSCs. The number of CFU-Fs present in mononuclear cells recovered from these fragments is of the same order as that obtained from medullar aspirations (1 CFU-F for $3.2 \times 10^6$ cells vs. 1–10 CFU-Fs for $1 \times 10^5$ cells) [13]. The MSCs obtained in this material have identical potential for differentiation compared to MSCs from bone marrow, but proliferation quickly decreases after the first phase of culture (L. Sensebé, unpublished data), which could be due to the age of donors.

Although bone marrow is the initial material of choice for culture of mesenchymal progenitor cells, related cell populations with similar properties are found in almost all tissues in mammals or humans [14, 15]. Thus, certain tissues such as fat tissue, fetal tissue and liquids (amniotic liquid, placenta, umbilical cord blood (CB), and Wharton’s jelly) become alternative sources of mesenchymal progenitor cells. Harvesting such tissue is much less harmful than harvesting bone marrow. Other sources are more difficult to access, e.g. lung and synovial tissue, or rarely considered, e.g. peripheral blood.

Fat tissue is an important source of cells linked to MSCs. These cells are commonly called adipose-derived stromal cells (ADSCs) or ASCs (used here, according to the International Federation of Adipose Therapeutics and Science). Fat tissue contains at least 100 times more MSCs than bone marrow contains MSCs. These cells also have a greater potential for proliferation [16]. Thus, we estimated that less than 1 g of fat tissue could allow for generating $10^5$ ASCs in approximately 14 days of culture. The differentiation and immunomodulatory potential of these cells is equivalent to that of MSCs [17–20]. In vitro and in vivo, ASCs show potential for differentiation in cardiac tissue and endothelial cells, which reinforces interest in their use [21, 22]. However, their phenotype is slightly different from that of MSCs since, contrary to MSCs, they express CD34 antigen [23]. This marker disappears during culture, which can explain why it is sometimes not detected. In the case of fat tissue, the sites currently harvested are subcutaneous, in the abdominal zone. The tissue is obtained by liposuction or biopsy. Work in the mouse seems to indicate qualitative differences depending on the sites of harvest.

The possibility of obtaining adherent stroma from the CB was reported for the first time in 1994 [24], but other laboratories could not reproduce the described technique. In 2000, Mingell and colleagues [25] obtained MSCs from CB in approximately 25% of harvests. These findings were later confirmed [26, 27]. However, MSCs cannot be isolated from all CB (60% at the maximum). The critical parameters for success are the time between harvest and the beginning of culture (15 h seems to be the maximum), the volume of CB (>33 ml), and the total quantity of mononuclear cells (higher than $10^8$ cells) [28]. After cryopreservation, the chances of success are even more reduced (0–19% [29]). Although MSCs have lower concentration in CB than in marrow (1 per $10^5$ cells vs. 1 per $10^6$ cells), they have a greater potential for proliferation [27, 28]. The potential for differentiation of CB MSCs in different tissues is also broad. After enrichment by depletion, CB MSCs have...
been found to differentiate not only in the mesodermal but also in the endodermal and ectodermal pathway [27, 30, 77]. The existence of accredited networks of CB banks and the possibility of great potential for differentiation could increase the use of CB to produce MSCs for clinical use, provided that the cells are isolated before cryopreservation.

Mesenchymal progenitor cells present in tissues or fetal fluids such as amniotic liquid, placenta or Wharton’s jelly in general have greater proliferation and differentiation potential than bone marrow MSCs [31–33]. They also seem to express certain markers of stem cells, such as Oct-4, more strongly [32]. Like ASCs, placental MSCs are able to easily differentiate in endothelial cells.

The presence of CFU-Fs in blood of adult mammals was shown at the beginning of the 20th century [34]. Contamination by fragments of conjunctive tissue, which explained this presence, was invalidated by experiments. However, the existence of circulating MSCs remains a discussed subject [35, 36]. Regardless, MSCs are in very restricted number in the blood and, moreover, are not found in all tested individuals. In humans, mobilization of granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) could increase their frequency, although not all studies has confirmed this possibility [35]. Charbord and associates [37] found MSCs mobilized by hypoxia in rat. Whether such pathological conditions can produce the same effect in humans is of interest. These circulating MSCs seem to have the same spectrum of differentiation as bone marrow MSCs.

An interesting study on humans showed osteoblastic circulating progenitors identifiable by their surface expression of osteocalcin [38]. These cells exist at much higher frequency than circulating MSCs described in other publications (1–5%). Moreover, this frequency is increased with significant osseous formation (with adolescence or fracture). Sorted cells are able to form bone in vivo. On culture, they seem to adhere only gradually, so non-adherent cells must be kept in culture during a long period. The exact flications of these cells compared to MSCs cannot be determined because the authors did not test other types of differentiation. Finally, even if peripheral blood is easy to access, it does not seem to constitute a source of MSCs suitable for therapeutic use. However, the presence of MSCs in peripheral blood of normal humans raises the question of the reason for their presence and the mechanisms. MSCs are also found in dental pulp or periodontal ligaments and synovia [39–41]. Because of their mesenchymal origin and differentiation potential, satellite cells in muscle must be considered mesenchymal progenitor cells as well [42].

The very broad distribution of mesenchymal progenitor cells and findings from recent studies suggest that such cells are located in the perivascular zone [43]. This could be explained by extensive migration with the formation of the vascular network during the life of the fetus. Mesenchymal progenitor cells may be in a specific niche, the cellular and molecular composition of which remain to be determined.

**Mesenchymal Cell Isolation**

The purification of mesenchymal cells by use of membrane markers is described elsewhere in this special issue [78]. For cells isolated from bone marrow and other fluids, the initiation of culture can start directly or after a simple centrifugation. For cells found in tissue, the isolation starts with enzymatic dissociation that releases the cells from the extracellular matrix. The commonly used enzyme is collagenase. After digestion, the cellular suspension is filtered to eliminate the non-dissociated pieces of tissue and cellular aggregates. For fat tissue, an additional step of centrifugation eliminates the mature adipocytes, which float. In general, the mesenchymal progenitor cells are then purified because of their property of adherence to the plastic. After initiation of culture, the cells are left to adhere for 24 h to 7 days. Then non-adherent cells are eliminated with a change of medium. This method is reliable and inexpensive. However, other cells such as macrophages and endothelial cells also adhere, necessitating a certain period of culture and/or successive passages for elimination [2]. The MSCs have a density of 1.073 which allows for enriching the cellular suspension by lowering it on a density gradient [44]. Medullar MSCs contain a subpopulation of small-sized cells (<7 μm) with fast renewal (so-called RS cells) and great potential for differentiation [45, 46]. RS cells are sorted after culture by use of a 10-μm filter. They have a cloning efficiency of approximately 90% but evolve in larger cells quickly if the cellular density increases [47]. These techniques are simple to perform and lead to a homogeneous population of cells with a fibroblastic type.

**Mesenchymal Cell Culture**

Here we describe mainly culture of bone marrow MSCs. We will indicate the specific conditions for cells of other origins when they differ. At the start of culture, MSCs appear as round and refringent cells. After a few days, the cells acquire a fibroblastic aspect (fig. 1a and c). MSCs are adherent cells with round and refringent cells. After a few days, the cells acquire a fibroblastic aspect (fig. 1a and c). MSCs are adherent cells with

**Oxygen Tension**

Cell culture at 20% of oxygen does not correspond to physiological conditions. In fact, oxygen tension is much lower in vivo and differs depending on the tissue and even the position in a given tissue. For example, in bone, oxygen tension may vary from 12.5 to 1%. Thus, MSCs are exposed to hypoxia in vivo. MSC culture under hypoxia enhances their proliferation and extends their lifespan [48, 49]. Hypoxia also increases extracellular matrix synthesis and organization, and modifies the...
expression of genes, mainly those involved in glycolysis and metabolism [50]. It also induces up-regulation of growth factors such as vascular endothelial growth factor (VEGF), heparin-binding endothelial growth factor (HB-EGF) or placenta growth factor. However, under hypoxia, the differentiation potential of MSCs seems to be reduced [48, 51].

**Cell Culture Density**

The seeding density is a critical parameter to ensure the optimal growth rate of MSCs as well as to maintain the differentiation potential. For initial culture (primo-culture), many protocols involved cell densities up to $200 \times 10^3$ cells/cm$^2$ [52]. Substantially lower densities are possible. A cell density of $50 \times 10^3$ cells/cm$^2$ has achieved a good rate of proliferation in primo-culture [53]; for the next passages, a cell density between $5$ and $7 \times 10^3$ cells/cm$^2$ was used [52, 54]. However, the most immature progenitors were achieved with very low culture density (from $1$ to $50$ cells/cm$^2$ [45, 55]). Increasing the cell density from $10$ to $1,000$ cells/cm$^2$ led to reduced expansion (from $500$ to $30$ times) with reduced cloning efficiency which fell from $36$ to $12$% CFU-Fs. These results could explain why in certain experiments, with cell densities of $6 \times 10^3$ cells/cm$^2$, the proliferation rate is quickly reduced and differentiation potential lost. When the surface and duration of the culture are kept constant, culture at $1,000$ cells/cm$^2$ gives 5 times more cells than that at $50$ cells/cm$^2$ [55]. At low cell-culture density, the consumption of medium and the culture surface required to obtain a reasonable amount of cells considerably increases the cost of experiments. Thus, for passaging, a cell density of $10^5$ cells per cm$^2$ represents an acceptable compromise, with a correct maintenance of MSC potentials and sufficient number of cells produced for study [53]. For ASCs, the cell densities for the primo-culture and for the following passages are lower [21]. We found it possible to sow ASCs at $4 \times 10^3$ cells/cm$^2$ in primo-culture and $2 \times 10^3$ cells/cm$^2$ for the next passages. For CB or peripheral blood, however, the cell densities of primo-culture are much higher ($10^6$ cells/cm$^2$) than that for the other tissues.

**Number of Passages**

Since MSCs are adherent and show contact inhibition, MSC culture, when confluence is reached, requires cell passage, whereby MSCs are detached from their culture support, generally by the action of a proteolytic enzyme (trypsin), and then sown on a new support at less density (see above paragraph). After one or more passages, one can obtain a great quantity of MSCs. Moreover, the culture conditions and the successive passages are enough to ensure the final purity of MSCs and the disappearance of other initially adherent cells (macrophages, lymphocytes, endothelial cells). However, these successive passages can alter the characteristics of MSCs. In rat, bone marrow MSCs retain their proliferative characteristics and differentiation potential until the 15th passage, but in humans the proliferation rate is reduced and the multipotential-
ty progressively lost with increasing cell passages [56]. These deteriorations are more marked with the age of the donor [8]. The aging of MSCs during the expansion could be related to telomere length [8].

**Culture Medium and Growth Factors**

The culture medium is a crucial element for obtaining MSCs. The basic medium used can have many formulations. The most-used media are \( \alpha \)-modified Eagle’s medium (\( \alpha \)MEM) and Dulbecco’s MEM (DMEM) with low content of glucose. However, other formulations are the McCoy medium, RPMI 1640 or Iscove MEM (IMDM).

From the beginning of MSC culture experiments, Caplan and colleagues [57] highlighted that the basic medium is not sufficient to ensure adhesion and the proliferation of MSCs. One or more supplements should be added. Initially, MSCs were cultivated with fetal calf serum (FCS). The batch of FCS must be evaluated carefully to allow for optimal growth of MSCs and can vary in composition and quality from lot to lot. MSCs may be decreased in number to a maximum of 5 times, and the multipotentiality could be reduced with certain batches of FCS [58]. The concentrations used vary from 10 to 20%. FCS provides the growth factors necessary for cell proliferation and the proteins for attachment. FCS can be replaced by human serum or an equivalent. Results of the use of human sera are somewhat contradictory: the effects are worse than or equivalent to that with FCS [59, 60]. The differences may be due to the autologous or allogeneic nature of the preparation [60, 61]. We have also found variable results with preparations from different suppliers. The addition of fibroblast growth factor 2 (FGF-2) to the human serum could mitigate this variability [62]. Another solution is to use the growth factors contained in platelets (platelet-derived growth factor (PDGF), EGF, VEGF, etc.), which are strongly mitogenic for MSCs and have been used since the 1980s [63–66]. The preparation can be obtained by activation of platelets, for example with thrombin, or simply by a cycle of freezing/thawing that destroys the platelets and releases growth factors [64, 67]. In our experience, this substitute for FCS is effective for MSCs and for ASCs. In general, the cells grow more quickly with this process but retain their general morphology (fig. 2).

However, the sera also contain indefinite factors that can influence the physiology of the MSCs or modify the gene expression. Thus, the ability to cultivate MSCs in a definite medium is of interest. In the 1990s, at least two teams described formulations allowing the growth of MSCs in such a medium [57, 68]. Unfortunately, the formulations do not contain molecules ensuring MSC adhesion, which necessitates adsorbing a protein of attachment (fibronectin) on the surface of the culture. Moreover, they do not contain growth factors which must be added to the medium. No major developments in formulations have been published since then.

As we noted previously, a certain number of cytokines active in MSCs include PDGF, EGF, transforming growth factor \( \beta \).
(TGF-β), insulin-like growth factor 1 (IGF-1) and FGF-2 [57, 68]. Among them, FGF-2 appears to be the major cytokine for MSCs. It stimulates the proliferation of every vessel of mesodermal origin. In culture, MSCs express the receptors for FGF-1 and FGF-2, and under all conditions of culture, FGF-2 increases the proliferation of MSCs, with growth into multiple layers. This effect begins at low concentrations (0.2 ng/ml) and reaches a plateau at 20 ng/ml. FGF-2 does not seem to increase the CFU-F frequency but does promote activity for proliferation rate. FGF-2 maintains the multipotentiality and could increase the frequency of tri-potential progenitors [56, 69]. Thus, during culture, it could select the more immature progenitors [69]. Surprisingly, FGF-2 seems not to be active when MSCs are cultured in serum-free medium [68]. FGF-2 is currently being used in a clinical trial to supplement FCS for the amplification of MSCs.

The other most potent growth factor is EGF, which enhances MSC proliferation both in serum-containing and serum-free conditions. The other most potent growth factor is EGF, which enhances MSC proliferation both in serum-containing and serum-free conditions. EGF seems not to be active when MSCs are cultured in serum-free medium [68, 70]. However, EGF seems not to be active when MSCs are cultured in serum-free medium [68, 70]. EGF also increases the migration of MSCs [71]. PDGF is a proliferation factor in serum-free medium, but not in FCS-containing medium, possibly because serum contains a large quantity of this factor. In human plasma enriched with platelet growth factors, PDGF AA, AB and BB are the major cytokines and seem to play a pivotal role in the proliferation of the mesenchymal progenitor cells [64, 65].

A discussion of the particular conditions of culture that allow for increasing the potential of MSCs is necessary. In 2001, Verfaillie and colleagues [72] described the multipotent adult progenitor cell (MAPC). The culture conditions were based on the medium described by Caplan and associates [57] and cell density based on the work of Prockop and coworkers (culture at low density) [55]. This culture technique allows for optimal proliferation of cells and maintenance or acquisition of a differentiation potential covering the 3 embryonic layers [72–74]. Another team added hypoxia (3% O2) to a similar culture technique [75]. This team also obtained cells that have a very strong capacity for proliferation but express very immature cell markers (Oct-4, rex-1 and hert) and have ectodermal and endodermal differentiation potential. Lastly, Prockop and coworkers [76] showed that prolonged serum deprivation allowed for selecting a population of very undifferentiated cells that express several embryonic markers. However, all these techniques have been difficult to reproduce in other laboratories.

In conclusion, mesenchymal cells exist in almost all tissues, with similar phenotype and very similar potential of differentiation. The techniques that allow for their isolation and cultivation are also very similar. Thanks to their properties, these cells are very promising tools for regenerative medicine.

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