Summary

Bone marrow (BM)-derived adult Mesenchymal Stem Cells (MSCs) have the capacity to differentiate into different cell lines in vitro. This capacity makes them a likely cellular source for clinical application in tissue repair therapies. In our laboratory we have recently discovered that immunostimulatory oligonucleotides of the PyNTTTTGT class are potent stimulators for MSC expansion both in vitro and in vivo. This suggested the possibility of clinical use of PyNTTTTGT oligonucleotides in tissue repair therapies either by increasing the amount of MSC obtained in vitro in a given time or by increasing the amount of MSC available in vivo for the natural tissue repair system. Regarding this, we have found that treatment of osteoporosis and neuropathic pain, with PyNTTTTGT ODNs in animal models results in considerable improvement. The multiple activities of the immunostimulatory oligonucleotides of the PyNTTTTGT class suggest that they act on a vital center for organic homeostatic maintenance which coordinates not only the immunological defense against the aggression but also the subsequent tissue repair process stimulated by the damage produced by the aggression itself and/or by the associated defensive inflammatory process.

Keywords: stem cell expansion, oligodeoxinucleotide IMT504, tissue repair
Introduction

Mesenchymal stem cells (MSC) or human bone marrow stromal stem cells are multipotent stem cells that can differentiate into osteocytes, chondrocytes, adipocytes, myocytes, hepatocytes, endothelial cells and neurons (1-13). MSC are classically cultured from progenitor cells present in the bone marrow (BM); however these progenitors are also present in other adult tissues, such as muscle or fat. MSC can be cultured to expand their numbers while maintaining their multipotency and can theoretically be then transplanted into an injured body site for tissue repair or seeded into a shaped scaffold in order to generate appropriate tissue constructs. Preclinical studies performed in different animal models of tissue damage have given support to these possibilities (14-25). In consequence, clinical trials using MSCs expanded in vitro as tissue repair medicines have been initiated (26-28). In spite of this excitement about the potential clinical uses of MSC, some concerns have also arisen (29) in the following fields:

Economic

MSC–based therapies may be very expensive if based on autologous cells because it would be necessary to obtain a biopsy from the patient, expand the cells in culture and perform the required quality testing before administering the cells for therapy. Also, it is not clear whether this procedure will generate sufficient cells at the needed time. An alternative is the use of allogeneic MSC. Undifferentiated MSC do not express immunologically relevant cell surface markers and do not elicit significant immune response after allogeneic transplantation (30, 31). Therefore, a donor could be chosen ahead and the cells qualified and stored so that they will be ready when needed for therapy. However, numerous aspects, mainly regulatory, should be solved in order to use allogeneic MSC in regular clinical treatments.

Reproducibility

Reproducible results using allogeneic MSC treatments are possible only if the materials used are constant and stable. However, each allogeneic MSC lot should be initiated from a different donor, a fact that may introduce a considerable variation among lots and exclude the possibility of establishing a master cell bank. On the other hand, the initial material (e.g. bone marrow aspirate) contains numerous cell types and the available procedures to isolate MSC can seldom produce a preparation containing 100 % stem cells except for that derived from a
single cloned cell. This implies a prolonged growth \textit{in vitro} that may result in genetic variability own to the mutation chance during DNA replication.

\textbf{Controllability}

Stem cell preparations originated from human sources may contain viruses and microorganisms of uncertain affiliations. Only a few of them with known pathogenic action can be assayed with specific tests. The presence of microbial contaminants can also potentially affect the therapeutic value of an MSC preparation. Potential contaminants can also be present in the fetal calf serum used during the expansion culture, a fact that should not be underestimated.

The rationale behind MSC delivery to promote tissue repair is based on the assumption that endogenous repair involving MSC expansion and mobilization from the patient’s own bone marrow (or other stem cell reservoirs) is insufficient in many pathological circumstances. A logical alternative to external cell delivery would be the use of a defined synthetic drug to promote expansion and mobilization of the patient’s own MSC. Availability of a drug like this will solve most if not all the above-described problems related to the therapeutic applications of MSC external delivery. Regarding this, we have recently discovered that immunostimulatory oligonucleotides of the PyNTTTTGT class augment the number of MSC progenitors both \textit{in vitro} and \textit{in vivo} (32) and their potential as agents in tissue repair medicine will be discussed in the next sections.

\textbf{Immunostimulatory Oligodeoxynucleotides of the PyNTTTTGT Class}

Immunostimulatory oligodeoxynucleotides (ODNs) are synthetic molecules that stimulate different kinds of cells of the immune system of animals. ODNs that are active on human cells are grouped into two major classes: a) CpG ODNs, characterized by the presence of at least one active site bearing an unmethylated CpG in a given context (33), and b) PyNTTTTGT ODNs, which have at least one active site bearing the sequence PyNTTTTGT in which Py is C or T and N is A, T, C or G (34). \textit{In vitro}, both kinds of ODNs act on B cells and plasmacytoid dendritic cells, causing activation, proliferation, immunoglobulin secretion and expression of co-stimulatory molecules, respectively. However, phosphorothioate CpG ODNs induce the secretion of IFN $\alpha$ (33), while phosphorothioate PyNTTTTGT ODNs do not (34). On the other hand, in the presence of IL2, PyNTTTTGT ODNs induce the secretion
of granulocyte macrophage-colony stimulating factor (GM-CSF) and IFN-γ acting on NK and NKT cells, while phosphorothioate CpG ODNs do not (unpublished data). Both CpG and PyNTTTTGT ODNs are good adjuvant in vaccines (33, 35) and promising anticancer agents (33, 36).

**PyNTTTTGT ODNs and MSC Culture**

MSC are generally cultured from progenitor cells present in bone marrow aspirates. During culture, these progenitor cells adhere to the plastic culture dish and form colonies of MSC. If a PyNTTTTGT ODN like the prototype IMT504, which is a phosphorothioate ODN (TCATCATTTGTACATT), is present during the first stages of the culture, the number of recovered MSC colonies is significantly increased when using BM cells from either rats or humans as starting material (Fig.1).

Fig. 1. Effect of IMT504 on MSC colony *in vitro* recovery from rat and human bone marrow. BM cells were seeded in tissue culture flasks containing α-MEM medium (GIBCO, Invitrogen Corp. USA) with supplements as described (32). Cells were fixed with methanol and stained using Giemsa’s azuroeosin-methylene blue solution (Merck, Darmstadt, Germany).

The effect of this ODN may be owed either to the stimulation of the replication or to the attachment of the MSC progenitors stimulated by soluble factors secreted by cells sensitive to the ODN present in the BM cell pool or to the direct or indirect inhibition of
apoptosis of such precursors. An anti-apoptotic effect on isolated normal B-cells has been previously observed (36). On the other hand, we have observed that differences in the total number of recovered MSC colonies increases as the total amount of fetal calf serum (FCS) added to the culture medium decreases (Fig.2).

![Graph](image)

**Fig. 2.** Effect of IMT504 and FCS concentration on MSC colony *in vitro* recovery from rat bone marrow. Deviation of the mean are expressed as SD. For 5 and 10% FCS concentration results were statistically significant (Student's t-test).

Since FCS has anti-apoptotic properties, this suggests the possibility that IMT504 may also have at least some anti-apoptotic effect on the MSC progenitors. Another important fact is that MSC resulting from IMT504-supplemented culture media preserve the multipotent differentiation capacity (Fig. 3). Therefore, PyNTTTTGT ODNs are very promising reagents to complement growth media for MSC production *in vitro* in order to obtain in a given culture time more cells with much less addition of FCS.
PyNTTTTG ODNs and In Vivo MSC Expansion

Even though the use of PyNTTTTGT ODNs to augment the number of MSC obtained \textit{in vitro} in a given time could be of great help to improve medical protocols of tissue repair using cell infusion, clearly more important is the possibility of rapid repair of tissue damage by raising the patient’s own MSCs by systemic treatment with a drug like the ODN IMT504 avoiding cell manipulation. In fact, experiments performed in rats suggest that this attractive option is possible. Upon subcutaneous injection of the animals with IMT504, MSC progenitors augment in bone marrow (Fig 4 A) as well as in circulation (Fig. 4 B).
Fig. 4. “In vivo” effect of IMT504 on MSC colony recovery from rat bone marrow (A) and blood (B). Differences were statistically significant (unpaired t test). PBM: Peripheral Blood Mononuclear.

**PyNTTTTGT ODNs and Tissue Repair in Animal Models**

1) Osteoporosis

Osteoporosis is a progressive systemic disease characterized by low bone mass and micro-architectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture. Due to its important prevalence worldwide, osteoporosis is considered a serious public health concern. While osteoporosis occurs in men and pre-menopausal women, the problem is overwhelmingly prevalent in postmenopausal women. Currently it is estimated that over 200 million people worldwide suffer from this disease. Approximately 30% of all postmenopausal women have osteoporosis in the United States and in Europe, and at least 40% of these women and 15-30% of men will sustain one or more fragility fractures in their remaining lifetime. Ageing of populations worldwide will be responsible for a major increase of the incidence of osteoporosis in postmenopausal women.
Osteoporotic fractures are those that occur under slight amount of stresses that would not normally lead to fractures in nonosteoporotic people. Typical fractures occur in the vertebral column, hip and wrist. The mechanism underlying osteoporosis is an imbalance between bone resorption and bone formation. Either bone resorption is excessive and/or bone formation is diminished. Bone is manufactured by osteoblast cells, whereas bone resorption is accomplished by osteoclast cells. Trabecular bone is the sponge-like bone in the center of long bones and vertebrae and cortical bone is the hard outer shell of bones. Trabecular bone is more subject to bone turnover and to remodeling and appears to be more affected by osteoporosis. The main histological alteration in the structure of the osteoporotic bone is a generalized thinning of the trabeculae. Common osteoporotic fracture sites, the wrist, the hip and the spine, have a high trabecular bone to cortical bone ratio and these areas rely on trabecular bone for strength.

Ovariectomy induces bone loss in the rat and postmenopausal bone loss share many similarities. These include increased rate of bone turnover with resorption exceeding formation and great loss of trabecular bone. Treatment of ovariectomized rats with IMT504 considerably improves bone quality (Fig. 5).

![Fig. 5. In vivo effect of IMT504 on femur recovery from ovariectomized (OVX) rats. Arrows indicate regions of increased bone density in treated OVX animals](image-url)
2) Neuropathic Pain

Neuropathic pain refers to a heterogeneous group of pain conditions characterized by lesion or dysfunction of the normal sensory pathways. Neuropathic pain is characterized by symptoms such as allodynia (pain due to a stimulus that does not normally provoke pain) and hyperalgesia (an increased response to a stimulus that is normally painful). Neuropathic pain is associated with a variety of etiologies, including trauma, infection, diabetes, immune deficiencies, ischemic disorders, and toxic neuropathies. Approximately 26 million patients are affected worldwide and the lifestyle of these patients can be severely impeded, a problem compounded by the lack of efficacy and frequent incidence of side effects associated with current treatment options. Opioids are frequently ineffective in treating neuropathic pain and current treatments, such as that with gabapentin (Neurontin TM), involve non-selective regulation of neurotransmitter systems or ion channels and generally result in significant dose-limiting CNS side effects. In this area where no current single drug treatment is effective in more than 50% of patients, novel therapeutic approaches are an urgent priority. In a neuropathic pain model (sciatic nerve crush in rats), subcutaneously injected IMT504 showed significant protective effects as revealed by two tests (Von Frey and Choi) which measure alldynia (Fig.6). The observed protection is similar to the one observed when animals are treated with an infusion of MSC at the site of the damage (38 ) suggesting that nerve repair and/or nerve environment repair at the site of the lesion is responsible for the protection observed upon treatment with IMT504.

![Fig. 6. In vivo effect of IMT504 on a sciatic nerve crush rat model of neuropathic pain.](image-url)
Perspectives

The multiple activities of the immunostimulatory oligonucleotides of the PyNTTTTGT class suggest that they act on a vital center for organic homeostatic maintenance which coordinates not only the immunological defense against the aggression but also the subsequent tissue repair process stimulated by the damage produced by the aggression itself and/or by the associated defensive inflammatory process. As illustrated by the above discussed examples, these properties of the PyNTTTTGT ODNs open, from the medical point of view, a number of perspectives:

- PyNTTTTGT ODNs can be used to complement growth media for MSC production in vitro in order to obtain in a given culture time more cells with much less addition of FCS, a critical component of the culture media (37)
- PyNTTTTGT ODNs can be used in combination with biomimetic scaffolds to increase the speed of in situ specific tissue development and
- PyNTTTTGT ODNs can be directly injected in patients in order to increase the speed of the natural tissue repair process.

On the other hand, several properties of the PyNTTTTGT ODNs make them very attractive for tissue repair medical procedures. Among these are:

- PyNTTTTGT ODNs are very easy to synthesize in vitro
- Due to its high solubility in water, PyNTTTTGT ODNs are very promising from the pharmacotechnical point of view
- In preclinical trials PyNTTTTGT ODNs have demonstrated to be very safe drugs
- Because PyNTTTTGT ODNs act on the patient’s own stem cells, they are completely free of immunological rejection, and
- Because PyNTTTTGT ODNs act on the patient’s own mesenchymal stem cells, they are free of the ethical issues typical of the use of embryonic stem cells.

Clinical trials will soon assess the possibility of medical use of these very promising chemical compounds.
References


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