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ADIPOSE MESENCHYMAL STEM CELLS AND "REGENERATIVE ADIPOSE TISSUE GRAFT" (LIPOGEMS™) FOR MUSCULOSKELETAL REGENERATION

C. TREMOLADA[1], G. BELTRAMI[1], A. MAGRI[1], F. BIANCHI[2], C. VENTURA[2,3], C. DI VITO[4], R. CAMPANELLA[5], S.E. NAVONE[6], G. MARFIA[6] and A.I. CAPLAN[7]

[1]Image Institute, Milan, Italy; [2]Cardiovascular Department, University of Bologna, Italy; [3]Laboratory of Molecular Biology and Stem Cell Engineering, National Institute of Biostructures and Biosystems (NIBB), Rome, Italy; [4]Department of Medical Biotechnology and Translational Medicine, LITA-Segrate, University of Milan, Italy; [5]Neurosurgical Unit, San Carlo Borromeo Hospital, Milan, Italy; [6]Laboratory of Experimental Neurosurgery and Cell Therapy, Neurosurgery Unit, Fondazione IRCCS CA' Granda Ospedale Policlinico Milano, Italy; [7]Skeletal Research Center, Department of Biology, CaseWestern Reserve University, Cleveland, USA

Regenerative medicine is a high-potential sector of strategic developments in medicine and health industry. The perspective to cure diseases up to now relied on medical treatments of long duration and limited effectiveness, and the possibility to avoid organ transplantation renders regenerative medicine attractive. In recent years, basic and translation research held great hope for this new field with significant progress in the modulation of stem cell commitment in vitro and providing protocols for targeted clinical applications. In line with this approach, mesenchymal stem cells (MSCs) have been introduced as potential therapeutic tools to correct the breakdown of musculoskeletal disorders. MSCs are able to secrete a large number of trophic factors capable of repairing the recipient tissue through angiogenic, anti-apoptotic and anti-fibrotic mechanisms. In this context, adipose tissue is emerging as a clinically relevant and easy to harvest source of multipotent progenitors to develop regenerative therapies. The present review focuses on the clinical application of MSCs, and in particular of adipose-derived stem cells, in the musculoskeletal disorders and on the current scientific challenges. In this perspective, we discuss future developments of an innovative system (Lipogems) for musculoskeletal regeneration, yielding a non-expanded and ready-to-use microfractured fat tissue product harbouring MSCs and pericytes within a preserved stromal vascular niche. The Lipogems system may also pave the way for future off-the-shelf and large-scale approaches for reconstructive procedures and regenerative medicine.

Musculoskeletal diseases are common conditions, including more than 150 different diseases. According to the World Health Organisation, these disorders are the most common cause of severe long-term pain and physical disability (1). With aging of the population, the incidence of musculoskeletal disease is rising and will be a significant socio-economic burden on society. The complex nature of these conditions generally means that treatment options are limited to managing symptoms, rather than prevention and cure (2). Therefore, there is an urgent need to develop new and effective therapeutic approaches for these age-related

Key words: musculoskeletal disorders, mesenchymal stem cells, stromal-vascular niche, lipogems

Corresponding author: Carlo Tremolada, MD,
President Lipogems International, srl,
Director Istituto Image,
v.le Bianca Maria, 24,
20129 Milan, Italy
Tel.: +39 3356963371 Fax: +39 02 3651834
E-mail: carlo.tremolada@gmail.com

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disabling pathologies. In this context, great strides have been made in regenerative therapies thanks to stem cell technology. More in detail, recent research has extensively shown the potential of mesenchymal stem cells (MSCs), a class of multipotent stromal stem cells, for reparative/regenerative medicine even in musculoskeletal system (3). Indeed, MSCs isolated from various tissues can differentiate into relevant cell types, thus representing an attractive option for cell-based therapy (4). Moreover, MSCs hold a great promise in tissue restoration thanks to their ability to secrete a wide range of growth factors, which have trophic effects on surrounding host cells, stimulating reparative responses (5). Furthermore, MSCs injected in pathological tissues contribute to favour physiological process by acting as reservoirs of repair cells or immunomodulatory sentinel to reduce inflammation (6). Several early-stage clinical trials are testing the delivery of MSCs in musculoskeletal disease, such as tendon injury, knee osteoarthritis, rheumatoid arthritis. However, despite the excellent potential of MSCs in regenerative medicine, many challenge must be overcome before they can be clinically used (7). In particular, it is essential to standardize protocols of isolation, expansion, and transplantation and to better understand MSC biology. The present review focuses on the clinical application of MSCs in the musculoskeletal disorders, and on the current scientific challenges, in which the difficulty of ex vivo expansion and the complexity of the current Good Manufacturing Practice (cGMP) requirements for expanded cells prompt the development of novel approaches in the autologous use of MSCs. In this perspective, we discuss future developments of an innovative system (Lipogems) for musculoskeletal regeneration, yielding a non-expanded and ready-to-use microfractured fat tissue product harbouring MSCs and pericytes within a preserved stromal vascular niche.

MESENCHYMAL STEM CELLS

Biology

Even if the exact location and function in the tissue of origin is not fully understood, mesenchymal stem cells are defined as multipotent and self-renewable cells with the ability in vitro to adhere to plastic and to differentiate into multiple lineages, including osteogenic, chondrogenic and adipogenic ones (8). Indeed, the International Society for Cellular Therapy define MSCs as a heterogeneous population of progenitor cells expressing a pattern of characteristic, but not specific, surface markers, including CD73, CD90, and CD105, but lacking the expression of hematopoietic markers CD34, CD45, CD14 or CD11, CD79a or CD19, and HLA class II (4). MSCs can be extracted from several body districts, including the adipose and synovial tissues, peripheral blood, skeletal muscle, umbilical cord blood, placenta, and bone marrow. However, an optimal source of MSCs in tissue regeneration and repair has not been yet identified. Indeed, MSCs deriving from different sources have similar, but not equal ability to differentiate into a specific kind of specialized cells. Moreover, the number of cells obtained may depend on the donor age and co-morbid conditions.

Secretoma and stromal-vascular niche

The participation of MSCs in tissue regeneration has been largely investigated according to the notion that these cells can themselves differentiate into some cell types, including bone, cartilage, muscle, adipocytes, stroma, fibroblasts and endothelial cells (9-11). Recent studies suggest that MSCs could participate in tissue repair, not only differentiating into cells of the target issue, but also releasing several factors, contributing to restorative processes, including angiogenetic onces (11-13). Indeed, the secreted trophic factors, participate to tissue rescue through pro-angiogenic and anti-fibrotic mechanisms (14-20), anti-inflammatory and immunomodulatory properties (21-29), anti-apoptotic and antimicrobial characteristics (30-38). Recent studies show a direct correlation between the occurrence of MSCs and the blood vessel density in stromal vascularized tissues (14,39,40). The niche is the morpho-functional unit where stem cells live and reproduce themselves. It is a particular kind of tissue within each tissue, in which a huge network of messages is fashioned through the product of the overall paracrine activity of the embedded cells, the so-called “secretome” (41,42). The regenerative potency of MSCs depends mostly on their ability to afford a timely modulation in the composition of the secretome (43). Further

understanding of the molecular pathways involved in growth factor production will be very helpful to develop better strategies for MSC-based therapies. In this new vision, the preservation of the niche is fundamental to consider MSCs as a patient-specific "molecular biology laboratory" adapting over time to the environmental cues released by the injured tissues (44).

Immunomodulatory effects

One of the most interesting characteristic of MSCs is their inherent immunomodulatory properties. When transplanted in vivo, MSCs do not elicit an immune response, allowing them to be used in allogenic stem cell therapy. In particular, they produce anti-inflammatory cytokines and suppress the proliferation, differentiation and function of immune cells in vitro (45). To take full advantage of the unique proprieties of MSCs for tissue engineering applications, a critical issue will be gaining further insights on the mechanisms controlling their self-renewal and differentiation, which will potentially result in the chance to modulate the behaviour of these cells for therapeutic purposes. Several characteristics of MSCs are purported to impart immune privilege, thereby allowing MSCs to avoid immune rejection in certain situations, which may facilitate the clinical use of allogenic MSCs. MSCs do not express class II Major Histocompatibility Complex (MHC) or costimulator molecules and express low levels of class I MHC (46). One of the first evidence of MSCs role in modulating immune reactions shows that activated MSCs inhibit T-cell expansion in mixed lymphocyte reactions (47). Moreover, MSCs influence the immune system through the secretion of a variety of soluble factors including indoleamine 2,3-dioxygenase (48), nitric oxide (49), transforming growth factor beta (TGF-□), prostaglandin E2 (PGE2) (50), and tumor necrosis factor stimulated gene- Protein (TSG-6) (51). Therefore, in addition to the direct regenerative effect of transplanted MSCs, these cells could interfere with microenvironment to stimulate physiological regeneration. However the precise mechanism of MSC action is not fully elucidate. A deeper comprehension of MSC biology both in vitro and in vivo could lead to the development of trustworthy strategies for clinical application of MSCs.

ADIPOSE MESENCHYMAL STEM CELLS

Adipose tissue is a connective tissue derived from embryonic mesoderm, consisting of a heterogeneous population of cells like adipocytes, preadipocytes, smooth muscle cells, endothelial cells, mast cells, fibroblasts immune cells. About the 10% of the adipocyte population is annually renewed (52). From adipose tissue manipulation it is possible to isolate the so called stromal vascular fraction (SVF), containing, among others, mesenchymal stem cells (53). SVF is easy harvested with minimal donor site morbidity commonly through lipoaspiration followed by in vitro cell isolation (54). The efficiency of SVF isolation is strictly related to donor general condition, such as age and obesity (55). In addition, compared to bone marrow, 1 g of adipose tissue contains 500 times more pluripotent cells than 1 g of bone marrow aspirate (54,56). Moreover, besides showing phenotypic and transcriptional profiles similar to that of the other MSCs, ADSCs present some peculiar characteristics. In particular, ADSCs express CD34 glycoprotein (57), stromal markers (CD13, CD29, CD44, CD63, CD73, CD90, and CD166) and endothelial cell markers (CD31, CD144, VEGFR2, and von Willebrand factor) (58). Moreover, ADSCs have a higher yield upon isolation and a greater proliferative rate in culture when compare with MSCs isolated from other sources (59,60). Of note, it has been demonstrated that these cells, besides the multiple mesodermal potential, are also able to differentiate in cells of ectodermal and endodermal origin, considering them as pluripotent stem cells (54,61). Therefore, adipose tissue represents a promising and clinically relevant source of multipotent progenitors to develop regenerative therapies. However, some challenges must be overcome before their use in tissue engeneering (7). One of the most relevant problem in the use of ADSCs is their expansion for several weeks in vitro prior implantation, that can modify their pluripotent potential and precluding their use in emergency circumstances (62). Moreover, emerging evidence indicates that culturing ADSCs the risk of infection, immunogenicity, genetic instability, and tumorigenicity could increase (63,64). Therefore, there is an urgent need to assess the safety of

ADSC implantation in humans, and to identify novel protocols for ADSC isolation, with minimal manipulation.

APPLICATION OF ADSCS FOR MUSCULOSKELETAL REGENERATION

The potential applications of MSCs in regenerative medicine, and, in particular, in musculoskeletal regeneration, show encouraging results for possible clinical applications. However, only few clinical studies use MSC-based approaches for such purposes, due to medical, research, and regulatory reasons (65). However, due to the lack of specific MSC markers, little is known on the in vivo ability of MSCs to differentiate. Indeed, different in vivo studies show that MSCs could participate to regeneration in various injury models, however, any evidence of clonal expansion, the true differentiation, and regenerative potential does not exists. Therefore, more studies are necessary to elucidate the mechanisms and biological properties of MSCs in determining their therapeutic efficacy in various diseases. Here we summarize the current application of MSCs for musculoskeletal diseases.

Cartilage repair

Cartilage repair represents a clinical challenging due to the intrinsic limited ability to repair itself. The current treatment of cartilage defects includes intra-articular administration of hyaluronic acid, treatment with platelet-rich-plasma, that contains bioactive proteins such as chemokines, cytokines and growth factors, and bone marrow stimulation, that include subchondral drilling, abrasion, and microfracture. Up to now, the only approved cellular-based therapy for cartilage restoration is based on the autologous chondrocyte implantation after in vitro expansion (66). Despite the promising initial results, the limited expansion of chondrocytes ex vivo, and donor site morbidity limit the beneficial effects of this technique (66,67). Alternative cellular therapies focused on progenitor cell populations bone marrow stem cells, that once transplanted ameliorated pain and movement ability (68,69). Recently, ADSCs are emerging as a less invasive source of progenitors that can be differentiated into chondrocytes in vitro in a 3-dimensional environment (70), in presence of growth factors (TGF-β superfamily) (71) and then implanted to restore cartilage tissue (72). Moreover, it has been observed that also uninduced ADSCs were able to fully restore cartilage in ear auricle defects (73) and in patello patellofemoral joints (74). These promising data suggest, therefore, that a minimal ex vivo manipulation of ADSCs, thanks to the intrinsic ability of these cells to adapt to their environment in vivo, could allow the development of an easy and effective clinical treatment to restore cartilage defects.

Bone regeneration

Bone graft is extensively used in a wide range of orthopaedic, plastic oral, dental, and neurocranial surgical procedures (75). However, the use of autograft bone is often associated to morbidity and scarce regenerative properties. Indeed the harvest of bone is associated to pain, haematoma formation, infections, and fractures (76). Moreover, aging reduce the availability of bone marrow stem cells (77). Therefore, research efforts in the field of orthopedics have been directed to ameliorate treatments for reconstructing bony defects, including traumas, tumors, infections, aseptic loosening or nonunions. These investigations have led to the development of improved treatments with strong osteogenic potency, as the recombinant bone morphogenetic proteins (BMPs). BMPs induce and promote critical steps of mostly endochondral bone formation and have been approved for clinical use in spinal fusion, non-unions and severely compromised long bone fractures. However, BMP technology has some therapeutic limitations when the microenvironment is compromised with poor or no vascularization. Therefore, to improve the therapeutic efficacy of this method, BMP administration has been successfully applied together with biomaterials and stem cells (78). In this regard, ADSCs, thanks to their osteogenic potential, have proven as good candidates both in vitro and in vivo (79,80). Moreover, growing evidence indicate that ADSCs alone, in absence of exogenous growth factors, are able to restore bone defects. In particular, it has been shown that ADSC transplantation could represent a good therapeutic option to treat craniofacial defects (81-83). Surprising results were obtained in the repair of the calvarium, that is unable to ossify after the first

two years of life. ADSCs, administered in presence of milled autologous cancellous bone and fibrin glue, or seeded in β-tricalcium phosphate granules, were able to completely repair calvarial defect, resulting in new bone formation and near complete ossification of the preoperative defect (81,83). However, the use of multiple concomitant treatments limits the comprehension of the therapeutic effect of ADSCs. Indeed, implanted cells could enhance bone regeneration through direct differentiation into mature osteoblasts and by paracrine effects, releasing osteogenic and vasculogenic molecules and growth factors, which facilitate migration and differentiation of resident precursors (84). Nevertheless the promising results obtained with ADSCs in bony defects restorations suggest that cell therapy represents an attractive alternative to traditional treatments like core decompression, osteotomy and total joint replacement, and provide a relatively simple method of autologous bony reconstruction with little donor site morbidity.

Arthritides

Arthritides, such as rheumatoid arthritis or osteoarthritis, are systemic and disabling autoimmune pathologies characterized by chronic joint inflammation and bone erosion. The crucial process underlying arthritides initiation is the abnormal activation of dendritic cells, T cells, B cells, macrophages, and neutrophils (85). The current treatments for these diseases are mainly addressed to manage symptoms and inflammation. However, a treatment that solves definitively the arthritides does not exist. Some studies focused their attention on cell-based approaches, taking into account that is a systemic defect, differently from focal articular defects, and the underlying disease process. In this context, MSCs, as previously mentioned, hold an immunoregulatory capacity, and elicit immunosuppressive effects, that can be used for several autoimmune diseases, including arthritides. In vitro studies on MSCs derived from umbilical cord demonstrated an anti-proliferative effect on synoviocytes, key players in inflammation and joint destruction in rheumatoid arthritis. This effect was mediated by the release of IL-10, 1-methyl-DLtryptophan, TGF-β1, and by the down-regulation of mixed metalloproteinase 9 (86). These results are in line with the in vivo studies on an arthritis rodent model, in which ADSC systemic injection reduced the levels of inflammatory cytokines and chemokines and the ratios of Th1/Th17 cells. Moreover, ADSC transplantation was able to increase the production of the anti-inflammatory IL-10 in lymph nodes and joints, and de novo generation of antigen-specific Treg cells (87,88). Similar results were obtained in the same mouse model after the intraperitoneal infusion of umbilical cord-derived MSCs: arthritis severity was reduced, the levels of proinflammatory cytokines and chemokines were decreased, and IL-10 levels were increased (86). Therefore, taking together these data suggest that ADSCs could ameliorate Arthritis pathogenesis decreasing the production of inflammatory cytokines and activating Treg cells. However, contradictory data was reported in adjuvant-induced and spontaneous arthritis model in which MSCs were effective only when injected before disease onset (89). Therefore, the anti-inflammatory function of stem cells may be effective in preventing or delaying arthritis if delivered at early stages of the disease process. More compelling investigations are needed to improve the specific targeting and the retention of MSCs at the cartilage surface in order to maximize their potential effect.

Tendon

Tendon injuries are often associated with significant dysfunction and disability, due to the limited self-repair capacity and propensity for scar formation of tendinous tissue. Despite the improvements in conventional treatment, such as transplant, clinical outcomes in tendon treatment are still variable. Moreover the use of allograft can lead to immune response and rejection (90), and the use of autografts is related to donor-site morbidity (91). Therefore, new strategies have been devised, such as tissue engineering techniques. Considering the fact that fibroblasts are involved in tendon healing by producing collagen, in an initial pilot study in humans, ex vivo expanded autologous dermal fibroblasts have been successfully used for refractory lateral elbow epicondylitis (92). Recently, with the progression in MSC characteristic identification, tissue engineering relying on these cells has been proposed to enhance tendon healing. In a rat experimental model of patellar tendon window defects, the injection of bone marrow stromal cells in a liquid fibrin matrix

stimulated histological, ultrastructural, molecular, and biomechanical parameters of patellar tendon healing, whereas injection of fibroblasts in fibrin matrix had only minor effects (93). Others in vivo studies showed that bone marrow MSCs were effective on tendon-bone healing. In a model of rat Achille's tendon damage, in which the enthesis (bone-tendon junction) was destroyed, the effect of chondrocytes or bone marrow MSCs were compared with not treated rats. Noteworthy, the bone marrow MSCs group showed an enthesis most similar to the premorbid state (94). Similar results were obtained in rabbits undergoing anterior cruciate ligament repair (95). These studies support the application of cell-based therapies for the regeneration of tendon tissues. However, these strategies have been investigated only in pre-clinical studies and the role of stem cells needs to be confirmed.

THE CHALLENGE OFFERED BY LIPOGEMS™-DERIVED ADSCs

Lipogems (PCT/IB2011/052204), represent a new completely closed tool to harvest, wash, process, and reinject human (or animal) lipoaspirates. Briefly, the surgical procedure consists in two steps: the infiltration step, in which adrenalin, in a saline solution, and very diluted lidocaine are injected to induce vasoconstriction and local anesthesia, facilitating the subsequent lipoaspiration; the aspiration step, in which a standard liposuction technique is performed. Afterwards, lipoaspirate is processed, by mild mechanical forces, passing floating adipose clusters through different reduction filters 59. Lipogems product is, therefore, a fat tissue derivative minimally manipulated that can be readily injected in an autologous fashion. The fact that Lipogems product is composed of completely normal pericytes (although beginning to detach from the vessels as in NORMAL response to trauma injury from the washing and cluster reduction process) and no cell expansion is done before injection is an extremely safe issue that make this technique just as a standard fat grafting but with less concern regarding the presence of oil and blood contaminations and with smaller size tissue clusters which makes the whole procedure less traumatic.

This device ameliorates the classical Coleman lipofilling technique (96), eliminating oil and blood residues lipoaspirate and reducing the size of the clusters of adipose tissue (97). These technical improvements have important biologic implications. In islet transplantation for diabetes treatment, the density of loading transplanted tissue and the size of clusters are important predictors of cell transplant outcome, in terms of inflammation, vascularization, and, therefore, in post-transplant engraftment (98). These promising results are probably due to the gentle fat tissue harvesting technique used in Lipogems. Differently from the lipoaspirate, which causes an imbalance in the cytoarchitectonics, Lipogems technique is not traumatic for cells and preserves vascular/stromal architecture with a high percentages of mature and a low amount of hematopoietic-like elements.

In an effort to characterized MSCs resident in fat tissue derivative, it has been observed that Lipogems-derived cells and lipoaspirate cells show a similar tissue architecture. Indeed, any differences in vimentin and fatty acid binding protein 4 distribution, as markers of adipose tissue, and in Ki-67 positivity, as marker of proliferation, were observed (99). Moreover, Bianchi and coworkers demonstrated that the in vitro culture of Lipogems derivatives allows the isolation of a population of ADSCs with a high degree of purity, than Lipoaspirate. These cells fulfilled the definition of mesenchymal stem cells, being able to differentiate mesoderm-type of cells, including osteogenic, chondrogenic, and adipogenic onces, like lipoaspirate cells (59). Nevertheless, intriguingly, Lipogems procedure was able to promote the expression of different antigens and to favour the multipotent potential with respect to lipoaspirate cells. In this regard, Carelli and colleagues demonstrated that Lipogems-ADSCs show a higher expression of self-renewal antigens, including OCT4, SOX2, NANOG, and of neural phenotype genes, such as ß-tubulin III, NEUROD1, PAX6, and SOX3 (99). Moreover, Lipogems-derived ADSCs, in response to pro-vasculogenic molecules, show a more pronounced expression of angiogenic genes, such as VEGF, KDR, and HGF (59). Furthermore, the treatment with a radio electric asymmetric conveyed field, was able to promote the transcription of genes involved in the commitment towards cardiac, vascular, neuronal,

and skeletal muscle lineages, and of stemness related genes, including Nanog, Sox-2, and Oct-4 (100). The possibility to obtain a final cell product containing viable adipocyte, preadipocyte, and stem cells, eliminating problems related to enzymatic digestion and other manipulations (8), exhibits a great appeal not only for its application in plastic and reconstructive medicine, but also in research, and regenerative medicine. Differently from lipoaspirate, the Lipogems product preserves the stromal vascular fraction also after cryopreservation, bypassing the difficulty of ex vivo expansion and the complexity of current Good Manufacturing Practice requirements for expanded cells. Of interest, the "earlier" cryopreservation of adipose tissue-derived stem cells represents an attractive challenge to treat different degenerative age-related pathologies, as younger adipose tissue progenitors and stem cells have a higher regenerative, tissue remodelling, and therapeutic potential (97,101,102). Based on its properties, the Lipogems product may pave the way to novel approaches and paradigms in the rescue of diseased tissues, within the context of both a personalized and a large-scale regenerative medicine (8).

CONCLUSIONS

Understanding the dynamics that regulate MSCs homeostasis, especially their anti-inflammatory effect and immunomodulatory capacity, has led to challenge a number of consolidated beliefs on their therapeutic mechanisms. Today, EMA (European Medicines Agency) and FDA (Food and Drug Administration), which had in the past significantly different views in conceiving stem cell biology, efficacy and storage conditions, have developed very similar rules. Nevertheless, these rules are constantly changing, and this is the reason why both Regulatory Agencies refers to "current Good Manufacturing Practice (cGMP)" as the set of guidelines and regulations that should dynamically ensure the currently best available standard for manufacturing medicinal products, including stem cells. However, the emergence of regenerative medicine raises new questions about the best ways to maintain quality. Regenerative medicine therapies involve new kinds of medical products, such as lab-grown or genetically modified cells. A therapy that uses living cells cannot be standardized in the same way as a conventional pill, and this makes sometimes very difficult to make statements and considerations that may remain valid for more than a few months (94,95). In this complex scenario, the challenge offered by Lipogems represents an attractive technique to obtain minimally manipulated fat tissue product that retains the intact microenvironment in which MSCs live, being amenable for the use in different clinical settings. Moreover, the multipotency of Lipogems-derived MSCs has been shown to be optimized by cell exposure or physical energy providing future off-the-shelf and large scale approaches for reconstructive procedures and regenerative medicine strategy.

DISCLOSURES. Prof. C. Tremolada is owner of patents and president of Lipogems International srl.

Prof. C. Ventura is a member of Lipogems International srl.

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