Biomedical Science and Engineering, 2014, Vol. 2, No. 1, 5-12
Available online at http://pubs.sciepub.com/bse/2/1/2

DOI:10.12691/bse-2-1-2

Different *in Vitro* Activation Methods for Latent Transforming Growth Factors (TGF)–β: Considerable Exogenous Factors to Promote Higher Mesenchymal-Origin Cell Proliferation in a Bioprocessing Platform

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Received December 16, 2013; Revised February 11, 2014; Accepted February 12, 2014

Abstract Regenerative medicine includes two efficient techniques, namely tissue-engineering and cell-based therapy in order to repair tissue damage efficiently. Most importantly, huge numbers of autologous cells are required to deal these practices. Nevertheless, primary cells, from autologous tissue, grow very slowly while culturing *in vitro*; moreover, they lose their natural characteristics over prolonged culturing period. Transforming growth factors-beta (TGF-β) is a ubiquitous protein found biologically in its latent form, which prevents it from eliciting a response until conversion to its active form. In active form, TGF-β acts as a proliferative agent in many cell lines of mesenchymal origin *in vitro*. This article reviews on some of the important activation methods-physiochemical, enzyme-mediated, non-specific protein interaction mediated, and drug-induced- of TGF-β, which may be established as exogenous factors to be used in culturing medium to obtain extensive proliferation of primary cells.

Keywords: *latent TGF-β activation, extensive cell culture, exogenous factors, hMSC proliferation, bioreactor*

Cite This Article: Partha S. Saha, and Michael Doran, "Different *in Vitro* Activation Methods for Latent Transforming Growth Factors (TGF)–β: Considerable Exogenous Factors to Promote Higher Mesenchymal-Origin Cell Proliferation in a Bioprocessing Platform." *Biomedical Science and Engineering*, vol. 2, no. 1 (2014): 5-12. doi: 10.12691/bse-2-1-2.

1. Introduction

Autologous cells, those cells which are collected directly from a subject itself, can offer maximum success to the practice of regenerative medicine. The most significant mesenchymal-origin cells having clinical applications in regenerative medicine are osteoblast, osteocytes, chondrocytes, myoblasts, tendoncytes, and ligament cells. Both streams of regenerative medicine call for huge number of viable autologous cells. Tissue engineering approach aims at populating these cells on 3D structure, and on the other hand, different types of bioreactors or micro-carrier cultivation techniques help to proliferate these cells within highly efficient, automated, robust and scalable culturing configurations and make the cells ready to be used for the clinical or industrial applications [1].

Scaling-up of the primary cells obtained from the autologous cells are among the major challenges for practicing tissue-engineering and cell-based therapy. This is because only very few number of primary cells can be harnessed from different tissues of a patient. For instance, number of mesenchymal stem cells (MSCs) present in bone marrow is about 0.01-0.001% of the total number of the mononuclear cells in bone marrow. Additionally, the availability of MSCs from bone marrow decreases with the advancement of the age of a patient [2]. Besides, these cells grow very slowly *in vitro*, and sometimes even tend to lose their actual genotypic and phenotypic entities over a prolonged culturing period, for example MSCs terminate to grow after 40-50 doublings [3].

Therefore, these limitations obviously indicate the necessity for a fast, robust, and reproducible cell-expansion technique to extensively produce clinically significant number of cells, in comparatively shorter periods of time with lower passage number. Adoption of systematic approaches in efficient systems may prove to overcome the bottlenecks of cell-expansion *in vitro*.

An effective method must be well-adoptable, cost-effective, and abundant for a scalable production of clinical graded cell *in vitro*. In those conditions, adopting the methods for activating latent transforming growth factors (TGF)-β (Figure 1), a well-abundant cytokine in cells, having relation to cell-proliferation, can be considered to be used in an *in vitro* culture to achieve higher proliferation of these slow-growing primary cells.

2. Transforming Growth Factors (TGF)

Transforming growth factors (TGF) are defined as those proteins (cytokine), which stimulate the growth of anchorage dependent non-tumorigenic cells [4]. Two classes of TGF can be found: TGF-alpha and TGF-β. Former can bind to the epidermal growth factor (EGF) receptor and also a mitogenic in soft agar medium [5].

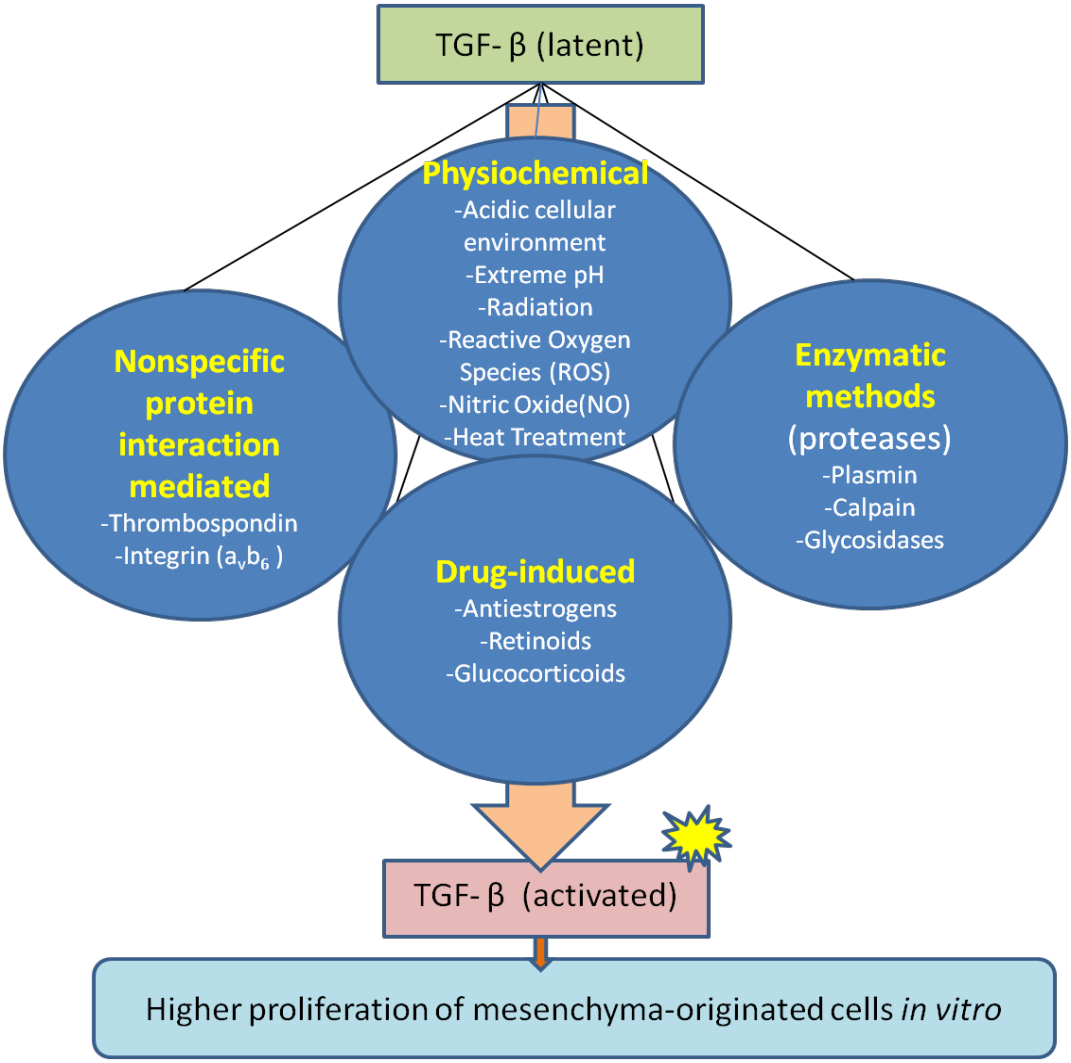

Figure 1. Different proteolytic and non-proteolytic mechanisms of activation of TGF-β *in vitro*

Unlike TGF-alpha, TGF-β is a significantly ubiquitous protein and has different functional and structural properties. TGF-β, however, is structurally and functionally different from TGF-alpha. TGF-β has its own cell surface receptors and mostly imparts a growth inhibitory action to most cell types [5].

TGF-β superfamily members mediate many key events related with the growth and development in mammals. These members have important roles in proliferation, differentiation, cellular functions, chemotaxis, tumor suppression, and apoptosis. They have also found to have effects on skeletal tissue development during the postnatal period as well as during development (reviewed in [6]).

TGF-β exists in five different isoforms, and among these only three are expressed in mammals, namely TGF-$\beta_{(1\text{-}3)}$. Despite their structural similarity, they have differential expression in mammalian tissue. Each of them binds to their respective TGF-β receptors with different affinities. Their distributions also suggest their different functionalities, for example 80%-90% of the TGF-β present in bone are in the TGF-β_1 isoform (reviewed in [7]); whereas, the prostate gland produces more TGF-β_2 than TGF-β_1 [8]. It is to be noted that in addition to different expression patterns, TGF-β/s have different biological effects *in vivo*, which mainly depend on the affinities of the isoforms for their receptors [9].

3. TGF-β Secreted as Latent Complex

TGF-β/s are found everywhere in the body, and its function is regulated not by means of their production and expression, but on its conversion from latent to active form [10].

Three different isoforms of the TGF-β are coded by three different TGF-β genes in chromosome of mammals. These three isoforms are synthesised as homodimeric proproteins (or pro-TGF-β) of mass 75 kDa. Latency associated propeptides (LAP), a dimeric propeptides, are cleaved from the mature TGF-β of 25 kDa molecular weight by furin type enzymes (reviewed in [11]). The LAP and the TGF-β are together attached by a non-covalent bond and form the small latent TGF-β complex (SLC). This SLC is joined with latent TGF-β binding protein (LTBP), and thus form LLC or large latent complex. LTBP, a protein of 125 to 160 kDa, includes different isoforms, ranging from LTBP-1 to 4. LTBPs

form a disulfide bond with LAP in order to localize LTBPs to ECM (reviewed in [10]).

4. TGF-β_1

TGF-β_1 is the most abundant and multifunctional isoform among all other isoforms of TGF-β. Platelets [12] and bone [13] are regarded as the largest source of TGF-β_1 in mammals. Some of the major functions of the TGF-β_1 include cell proliferation, cell differentiation, cell survival, and production of extracellular matrix (ECM). Therefore, TGF-β_1 is also involved with angiogenesis, wound healing, immune response and development, and bone formation. The bone formation by the TGF-β_1 is promoted by its activity on the enhancement of osteoblast proliferation, production of the ECM proteins, chemotactic attraction of osteoblasts, chondrocyte precursor cells to stimulate of type-II collagen expression and proteoglycan synthesis, as well as the suppression of proliferation of the hematopoietic precursor cells. Overall, this isoform has an extensive role in mammals, including embryogenesis to tissue homeostasis [14].

5. Effect of TGF-β on hMSCs

TGF-β_1 is known to promote human mesenchymal stem cells (hMSC) proliferation in presence of other growth factors [15]. It is to be also noted that TGF-β_1 alone has a growth-inhibitory effect on MSCs, and in presence of bFGF, the inhibitory effects become higher [16]. In addition to that TGF-β_1 plays a vital role in synthesis and degradation of different bone marrow complexes (e.g. collagen and non collagen proteins). But at the late stage of osteoblast differentiation, it inhibits the process [17].

According to James *et al*., TGF-β plays a key role in determining the fate of a MSC either to remain in an undifferentiated state or to be differentiated into a particular lineage [18]. It was found that in cooperation with Wnt and β-catenin signalling pathways, TGF-β promotes the chondrogenic and osteogenic differentiation respectively [19].

6. Role of TGF-β in Osteogenic Differentiation of MSCs

MSCs are clinically used for the treatment of bone defects. Therefore, it is necessary to understand the factors that regulate the differentiation and the proliferation of the MSCs. It is to be noted that TGF-β is one of those factors. TGF-β_1 is the main isoform that is present in the bone tissue as small latent complex. Again, TGF-β_1 is present in the undifferentiated MSCs. However, the latent TGF β_1 has no effect on the differentiation of the MSCs; as a result, the proliferation of the MSCs continues. This supports the involvement of the LTPB dependent mechanism for the differentiation of the MSCs to osteoblasts [20]. Among the isoforms of LTBP (LTBP-1 to 4), mainly LTBP-3 mediates the activation of the TGF-β complexes and stimulates the proliferation of MSCs. However, the down regulation of the LTBP-3 reduces the MSC proliferation [21].

7. Effects of TGF-β on Bone Cells

TGF-β has different effects on the different stages of osteoblast formation. In the early stage, TGF-β stimulates the proliferation of osteoblasts, while in the late stage, it inhibits osteoblast differentiation and mineralization in order to reduce the bone formation [22].

In vitro, TGF-β also stimulates a number of bone cells which have an osteoblast phenotype [23]. TGF-β also stimulates alkaline phosphatase activity, osteonectin, collagen synthesis and osteopontin synthesis [24]. TGF-β, in addition, is involved with the formation of ECM by stimulating different protease inhibitors [25].

However, the *in vitro* action of the TGF-β on the bone cells is found to be contradictory. This is because the effect of TGF-β on the bone cells in a culture largely depends upon different culture factors, such as cell density, TGF-β concentration, differentiation stage of the cells, and the culture condition [9].

It is known that TGF-β has a dramatic effect on the process of bone remodelling [14]. After being released and activated by the osteoclasts, TGF-β expands the pool of committed osteoblasts by recruiting the precursor of the osteoblasts and stimulates them to proliferate. Several reports have shown that they have a mitogenic effect on the osteoblastic precursors [26,27].

It has been mentioned before that TGF-β has a stimulatory effect on the osteoblastic differentiation only in the early stages, but in the late stage it has inhibitory action on the mineralization process. This is because of the fact that during the transition from the pre-osteoblast to matured osteoblast, there is a decreased expression of the TGF-β receptors [28]. Additionally, they are also involved in formation of the ECM or extracellular matrix in numerous cell types. Conversely, TGF-β does not have any osteoblastic differentiating effect on the uncommitted MSCs, yet it affects only the committed MSCs [29].

On the contrary, TGF-β also regulates different stages of the osteoclast resorption in a similar fashion to the osteoblast-regulation.

8. Proliferative Effects of TGF-β *in Vitro*

The intrinsic actions of TGF-β on different cell systems have been categorised into proliferative, antiproliferative, and non-proliferative. Despite its name, TGF has found to be a growth suppressor of the proliferation of epithelial hematopoeitic and endothelial cells. However, in many cell lines of the mesenchymal origin, TGF-β acts as a proliferative agent (reviewed in [30]). One such type of cells is osteoblasts because they have a high affinity for TGF-β receptors, and they also respond to the exogenic TGF-β [31]. Besides increasing their own expression, TGF-β also helps to express the action of several other growth factors, such as platelet derived growth factor (PDGF), vascular endothelial growth factors (VEGF), basic fibroblastic growth factor (bFGF). This increased expression induces cell proliferation in an autocrine mode [32,33].

9. Activation of Latent TGF-β

9.1. The Interaction between LBP and TGF-β Can be Disrupted by Physiochemical and Enzymatic Treatments

Since TGF-β signalling receptors are ubiquitously expressed, therefore, the activation of TGF-β is a key step in regulating many biological effects. This activation can only be achieved through the activation of TGF-β from its latent form to active form.

On the other side, latency is a mechanism to prevent the cytokines from eliciting a response until conversion to the active form. Generally, mature TGF-β remains in association with the LAP, a propeptide, through a noncovalent interaction. In order to elicit the biological activity, TGF-β must be released from the mentioned interaction. This process is called a TGF-β activation.

The interaction between TGF-β and LAP can be disrupted *in vitro* by physiochemical and enzymatic treatment. The denaturing condition, exerted by the physiochemical treatment is resisted better by TGF-β as compared to LAP. Therefore, this difference is used to disrupt the interaction between TGF-β and LAP [34]. Besides the commonly used heat treatment at 100°C and extreme pH treatment with 2 or 8, chaotropes or detergents like SDS and urea are also used to promote the activation (reviewed in [35]).

Latent TGF-β can also be activated *in vitro* by oxidation with free radicals and reactive oxygen species (ROS) [36]. Moreover, there are two enzymatic processes involved with the activation of TGF-β-firstly, treatment with enzymes, such as endoglycosidase, neuraminidase, sialidase, etc., and secondly, the proteolytic degradation of LAP by plasmin (serine protease), calpain, and cathepsins B and D. These release the activated TGF-β from the latent complex [35]. In addition to enzymatic activation, the glycoprotein, trombospondin-1 (TSP-1), and adhesive protein on cell surfaces and ECM interact with the latent TGF-β and activates it non-enzymatically [37]. It has been found that the addition of TSP-1 to the culture of endothelial cells can yield more activated TGF-β [38].

9.2. Mechanisms of Activation

The LLC, which is originally secreted from the cells, needs to be processed to release active TGF-β [10]. This release of TGF-β determines the level of free TGF-β in a medium. Therefore, the prime concern is the characterization of the mechanisms which can liberate the TGF-β from the latent complex [11].

The activation of the TGF-β can occur either via *in vitro* or *in vivo*. *In vitro* activation involves the use of direct activators; whereas, the *in vivo* and cell culture system involve the preliminary steps in activation and thereby activation *per se* [39].

9.2.1. Physiochemical mechanisms

9.2.1.1. TGF-β Activation in "Acidic Cellular Microenvironment"

In 1989, Jullien *et al*. found that acidification of culture with an agar-gelled over-layer, containing untransformed NRK-49F cells or KiMSV-transformed NRK-49F cells in the presence of FCS (a source of TGF-β), to a pH of 5.0 had elicited an EGF-dependent colony formation. In another experiment, they observed that the same result was obtained when they added pure, active TGF-β. Thereby, they concluded that activation of the latent TGF-β in the presence of transient acidification was the probable cause of the enhanced growth of the cells. In other experiment, they found that in the absence of the FCS, the positive growth result was obtained in response of EGF and active TGF-β [40].

9.2.1.2. TGF-β Activation in Extremes of pH

The non-enzymatic activation of TGF-β was shown by Lawrence *et al*. in an experiment with chicken embryo fibroblasts. In that experiment, the pH dependency of the activation of TGF-β, which was secreted by the chicken embryo fibroblasts, was found. In other words, with the increase of the acidity of the medium, the activity in terms of the cloning efficiency increased. It had been suggested by the authors that with the increase of the acidity, the higher molecular wt TGF-β changes into the low molecular weight form. Furthermore, it was found that the alkalization beyond pH 9.0 followed by a neutralization activates the TGF-β. In this case, it was suggested that once activated by the acidification, TGF-β can reform its original structure under neutral condition without causing any alteration to its activity [41].

Besides the native form of TGF-β, the recombinant forms of TGF-β are in the biologically latent complexes. It had been found by Brown *et al*. in 1990 that these latent forms of TGF-β can be shifted to the activated forms by putting them in a range of extreme pH. They observed that over a pH range of 4.1 and 3.1, the transition occurs in case of the latent forms of TGF-β_1 and TGF-β_2. On the other hand, they also observed that over a pH range between 3.1 and 2.5 and between pH 10.0 and 12.3, the activation of the chick recombinant latent TGF-β_3 occurred [42].

It was found by Lawrence *et al*. and Moses *et al*. that acidification at pH 1.5 for 1 hour, followed by neutralization resulted in activation of significant amounts of active TGF-β [41,43]. Similar conditions appeared when a conditioned medium, with both AKR-MCA and NRK-49F, was observed to enhance the production of the active TGF-β. Again, it was observed that there existed a two-fold difference in the amount of the acid-activated TGF-β secreted by the both types of cells in their respective medium [5].

9.2.1.3. TGF-β Activation with Radiation

It was observed by Barcellous-Hoff in 1993 that exposure of mouse mammary gland with gamma radiation induced immunoreactive TGF-β within 1 hour post-radiation. This activation of the TGF-β was thought to occur due to the induction of ROS [44].

In 1996, the same author and his colleague investigated the potential of the redox-mediated LTGF β activation *in vitro*, where ROS was generated in a cell free system, using ionizing radiation. It was found that an exposure of an irradiation (100 Gray) to recombinant human LTGF-β in a solution induced 26% more activated TGF-β as compared to the commonly used thermal method of TGF-β activation. From that observation, it was postulated by them that the release of the TGF-β happened due to the conformational change in the latent complex, which, in

turn, occurred due to oxidation of specific amino acid (s) in the latency-conferring peptide [45].

9.2.1.4. TGF-β Activation with Reactive Oxygen Species (ROS)

In 1996, Barcellos *et al.* studied that an irradiation with 50-200 Gray to iron containing saline resulted in generation of active TGF-β. The ionizing radiation generates a number of ROS and hydroxyl radicals, which do not actually affect TGF-β but affect LAP. In another experiment, they found that oxidation of ascorbate when catalyzed by metal, such as Fe^{3+} or Ca^{2+} in solution, activated recombinant LTGF-β very efficiently so much so that the activation efficiency almost matched or exceeded the commonly used thermal method of activation. The author furthermore found that ROS affects one or several amino acid residues, such as cysteine or methionine, which were susceptible to the oxidation. This led to the alteration of the stability of the LAP - which resulted in either LAP losing its noncovalent association with TGF-β or undergoing conformational change to expose the TGF-β receptor binding sites. Therefore, the oxidative activation with ROS endows the L-TGF-β the ability to act as a sensor of oxidative stress to function as signal [36].

9.2.1.5. TGF-β Activation with the Treatment of Nitric Oxide (NO)

Vodovotz *et al.* determined the effect of NO on latent TGF-β_1 *in vitro*, by exposing a culture of A549 human lung carcinoma cells to DEANO, a synthetic donor of NO. This treatment of cells with DEANO resulted in induction and activation of latent TGF-β_1 in a dose dependent manner. This exposure of the NO led the activation of latent TGF-β_1 bound at or near the cell surface. According to the author, this activation of latent TGF-β was due to some indirect approach because no appreciable amount of the active form of the TGF-β_1 could be detected even after 24 hours of treatment of the cells with DEANO [46].

9.2.1.6. TGF-β Activation with Heat Treatment

It was found by Brown *et al.* in 1990 that the native and recombinant latent form of the TGF-β_1, TGF-β_2 and TGF-β_3 can be activated with an exposure to heat in the ranges of 75-100°C and 65-100°C, respectively. However, the complete activation for the TGF-β_1 occurs at 80°C for 5 minutes of exposure; whereas, in the case of TGF-β_2 and TGF-β_3, the complete activation occurs at 100°C with a similar period of time [42].

With an aim to make a comparison between the effectiveness of the acidification and the heat treatment methods to activate TGF-β_1, Zamora *et al.*, carried out an experiment in two samples, containing 5000 pg/ml latent TGF-β_1. One of the groups was treated with 1N HCl, followed by neutralization by 1.2N NaOH. Another sample was incubated at 80°C for 5 min in a water bath. Thereafter, the level of activated TGF-β was measured using an of ELISA kit. It was concluded from the experiment that the heat treatment was more effective than that of the acidification method [47].

It is to be noted that the activation by the acidification, alkalinisation or chaotropic agents *in vitro* involves some discrepancies [47]. This discrepancy is just because of at least two different pools of latent TGF-β_1 in a conditioned media. One pool is resistant to the mild acid or plasmin, and takes the strong acid or alkali treatment for the activation. On the contrary, another pool may be prone to activation only by the mild pH change and/or plasmin [5].

It has been found that activation of TGF-β obtained by plasmin treatment of the medium is quantitatively similar to that of mild acid treatment. In addition, by adding anti-TGF-β antibodies the plasmin-generated activity could be inhibited. Interestingly, the sequential treatments with mild acid followed by plasmin or *vice versa* can give activation comparable to either treatment alone [5].

9.2.2. Enzymatic Methods of TGF-β Activation

9.2.2.1. TGF-β Activation with Proteases

9.2.2.1.1. Plasmin Mediated TGF-β Activation

In order to determine the potentiality of plasmin, a wide spectrum serine protease, to activate latent TGF-β, Lyons *et al.* used a culture media, containing cells: AKR-MCA mouse fibroblast and NRK 49F rat fibroblast. It was found that the plasmin activated the same pool of latent TGF-β, which was activated at pH 4.5 by acidification. It was concluded that the mild acidic condition was unable to activate latent TGF-β alone as the Arg-Arg site could not be cleaved, and this task was done by the plasmin. It is believed that the acidic condition stimulates the protease activity, which promoted the cleavage at another site of the structure [5].

In another set of experiments, the author compared the acid and plasmin activation of latent TGF-β in an AKR-MCA cell culture in a soft agar medium, in triplicate, in terms of number of colonies formed. From there, it was observed that with the increase of the plasmin concentration in the medium, the number of colonies formed increased. That, in fact, hinted for the activation of the latent TGF-β in the medium [5].

In a proposed model of the TGF-β activation by plasmin, it was suggested that the plasmin was activated by the extracellular plasminogen activator (PA). The activated plasmin then activates the inactive TGF-β proteolytically at or near the cell surface and thus allows the binding of the activated TGF-β to its receptors. This binding again enhances the production of the endothelial-type PA inhibitor and decreases the extracellular activity of urokinase-type PA (uPA). This initiates a negative feedback control of the TGF-β action [5].

9.2.2.1.2. Calpain Mediated TGF-β Activation

Calpain, one of the neutral cysteine proteases, has various physiological phenomena as a modulator. This protease was found to activate latent TGF-β [48].

The recombinant latent TGF-β was incubated with a concentration of 1-10 μg/ml, and found that LAP was freed from the latent complex by cleavage. The mature or active TGF-β form was detected by SDS-PAGE method and quantified by measuring its lucrifase activity (%). Later on, in another set of experiment, the authors observed that when bovine capillary endothelial (BCE) cells were cultured in the presence of low concentrations of calpain (i.e. 0.1 ug/ml), the proliferation of the mentioned cells was inhibited. However, most importantly, this inhibitory effect of the calpain was not observed with the addition of anti-TGF-β antibody and calpain inhibitor

peptide. Therefore, from here, it could be understood that active TGF-β, which is formed upon application of the calpain in low concentration, was involved with the cell proliferation [48].

9.2.2.1.3. TGF-β Activation with Glycosidases

According to Miyazono and Heldin, certain carbohydrate moieties present on LAP renders the latency of the L-TGF-β_1 *in vivo*. They also reported that, *in vitro* removal of this carbohydrate moiety is possible by enzymatic means. This removal of the carbohydrate structure presents active TGF-β from its latent complex [49]. Therefore, it can be concluded that the carbohydrate structures play an important role in the interaction of LAP and TGF-β. It is considered that competing concentrations of sialic acid and MP6 are effective to activate the latent TGF-β [49]. It has been found that this sialidase activity found in the activated macrophages uses this mechanism [39].

9.2.3. Non-Specific Protein Interactions for TGF-β Activation

9.2.3.1. Thrombospondin Mediated TGF-β Activation

TSP is a glycoprotein and present in five isoforms. Among these isoforms, TSP-1 is studied most. However, it has been found that when the LAP of L-TGF-β interacted with the Riboflavin kinase (RFK) sequence on the TSP-1, a conformational change took place. This conformational change exposed the TGF-β receptor binding site of the L-TGF-β, resulting in the interaction of the TGF-β to its receptor. Moreover, it had been found that this activation of the TGF-β complex did not require any presence of the plasminogen system or cell localization [38].

The activation of latent TGF-β was first found by Schultz-Cherry and Murphy-Ullrich. It was observed, when BAE cells were grown in addition of increasing concentration of the native TSP (TGF-β activity associated with TSP) and sTSP (no associated TGF-β activity) in presence of 1.5% FBS, there was an inhibition of the BAE cell growth, irrespective of the nature of the TSP [38].

It was investigated and found that not the increased or decreased secretion of TGF-β, but the activation of the endogenous TGF-β caused the inhibition of the cell growth. This was further proved by counting the decreased number of reduced colony formation by the NRK cells in soft agar with the conditioned medium. In addition to that the authors also found the activation of the recombinant TGF-β by using sTSP [38].

9.2.3.2. Integrin a_vb_6-Mediated TGF-β Activation

Integrins are dimeric cell surface receptors, which are composed of two subunits: alpha and β. The a_vb_6 was the first intergrin found to activate TGF-β. The direct interaction of the RGD amino acid sequence present in LAP-β_1 and LAP-β_3 with the a_vb_6 is found to activate the TGF-β complex [50]. However, the expressions of a_vb_6 receptors are mainly restricted to epithelia [51]. In response to any wound and inflammation, the expression of TGF-β is enhanced. Physiologically, epithelial cells activate TGF-β, by increasing the a_vb_6 production by epithelial cells in response to any wound and inflammation.

So as to show the formation of active TGF-β with the application of the a_vb_6 integrin, Munger *et al*. cocultured the mink lung epithelial reporter cells (as reporter cells) in the presence of the four different types of β6-expressing cells. A significant increase of the luciferase activity was observed, when the result was compared with the control. Most importantly, this increase was found to be inhibited with the application of the MAb against active TGF-β and a_vb_6. For this data, it could be concluded that a_vb_6 integrin produced active TGF-β [50].

Recently, it was also found by Mu *et al*. that the presence of the activity of a certain protease (MT1-MMP) was essential for the activation of latent TGF-β with the integrin a_vb_6. The author suggested that this protease concentrated the latent TGF-β on the cell-surface, where it was activated by a_vb_6 in the presence of MT1-MMP [52].

9.2.4. Drug-Induced TGF-β Activation

9.2.4.1. Antiestrogens Mediated TGF-β Activation

MCF-7 cells, a human breast cancer cell line, secrete a variety of polypeptides which have growth-promoting potential, such as IGF-I related growth factors, TGF-α, and TGF-β. The presence of active TGF-β was determined by determining the number of colony formations by the NRK-49F cells in a serum-free conditioned medium in the presence or absence of EGF [52].

It was found that upon treating MCF-7 cells with growth inhibitory concentrations of antiestrogen, the secretion of the active TGF-β was induced 8 to 27 folds [52].

9.2.4.2. Retinoids Mediated TGF-β Activation

The retinoids are regarded as important regulators of cell growth and differentiation. This is due to the increased expression of the active TGF-β from a culture of keratinocytes *in vitro*. This was proved by Glick *et al*. when they cultured primary keratinocytes treated with retinoic acid in serum free medium. They found the presence of a significant amount of active TGF-β in the conditioned medium in contrast to the untreated medium. Moreover, they found that the level of TGF-β increased with the increase of time (days). On the other hand, they also found a dose-dependent relationship between the increased concentration of the retinoic acid and the secreted TGF-β. The presence of the active form of TGF-β was indicated by formation of large numbers of colonies by the retinoic acid-treated keratinocytes in the presence of EGF within a specific period of time [54].

9.2.4.3. Glucocorticoids Mediated TGF-β Activation

It was found by Oursler *et al*. that dexamethasone, a potent glucocorticoid, had an influence over the secretion and activation of the latent TGF-β secreted by human osteoblast-like (hOB) cells. A dose-dependent relationship was observed between the activation and the given dose of this steroid. It was found that up to 90% of the TGF-β could be activated by this process. Even the conditioned medium (without cells) prepared from the dex-treated hOBs, was found to retain the TGF-β activation ability when incubated at 37°C. However, it was also observed that this activity was preventable upon treating the hOBs

with protease inhibitor, which was thought to disrupt the microtubules. The actual mechanism involved in the activation of the TGF-β was by increasing the levels of mRNA of cathepsin-B and –D, and also in the level of cathepsin-B, a lysosomal protease. These secretions of the lysosomal proteases activated the latent TGF-β, which was actually secreted by the hOBs. This is the way with which bone cell functions are regulated by the glucocorticoid [55].

10. Conclusion

Maximizing cellular proliferation by using exogenous factors can be economical as they may limit the use of expensive materials, having partial roles in cell culture. These different methods of activating latent TGF-β mentioned may be adopted to harness large cell population in suitable bio-processing setups, such as bioreactors. Since all these experiments were performed in different culture-environments, so it is difficult to state about the most efficient method among them. Nevertheless, taking other relevant cell-culturing techniques in considerations, if these methods are adopted alone or in combination, there may be a breakthrough in obtaining higher expansion for the cells, which have mesenchymal origin and also for others if applicable.

Acknowledgements

I, as a first author, am dedicating my contribution to Professor Dr. Kirsten Van Landuyt, KU Leuven BIOMAT, University of Leuven & Dentistry, Leuven, Belgium for her excellent supports and inspirations to pursue my interests in rigorous scientific researches. I am also thankful to Dr. Michael R. Doran, QUT, Brisbane, Australia and Dr. Iain R. Gibson, IMS, University of Aberdeen, United Kingdom, whose sincere help takes the form of this scientific publication.

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