Universidad de Navarra

Effect Of P144® (Anti-TGF-β) In a “In Vivo” Human Hypertrophic Scars Model In Nude Mice

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C E R T I F I C A : Que Shan Shan Qiu , licenciada en Medicina, ha realizado bajo mi dirección el trabajo de su tesis doctoral titulado: "Effect Of P144® (Anti-TGF-β) In an "In Vivo" Human Hypertrophic Scar Model In Nude Mice"

Revisado el presente trabajo, quedo conforme con su presentación para ser juzgado para optar al Grado de Doctor.

Y para que así conste y surtan los efectos oportunos, se expide el presente certificado en Pamplona a veintiuno de diciembre de 2015

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Effect Of P144® (Anti-TGF-β) In a "In Vivo" Human Hypertrophic Scars Model In Nude Mice

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SUMMARY

Background

Hypertrophic scars are one of the most relevant complications in surgery due to their cosmetic and functional impairments. Among the cytokines responsible of this anomalous healing, Transforming Growth Factor-beta (TGF-β) has been demonstrated to be one of the most representative. Previous studies in tissue fibrotic disorders have shown promising results by inhibiting the biological activity effect of TGF-β. The objective of the current study is to determine the effect of the inhibition of TGF-β1 signaling in human hypertrophic scars implanted in nude mice by topical application of a TGF-β1 receptor type III inhibitor peptide (P144®).

Material and methods

30 human hypertrophic scars were implanted in 60 mice. The animals were divided into two groups, group A (placebo) and group B (treatment). Group C (basal) was considered as the preimplanted scar tissue samples. When all the xenografts were shed, topical application of a lipogel containing placebo (group A) or P144 (group B) was daily administered during 15 consecutive days. After the completion of the treatment, the animals were sacrificed and the total area, scar thickness and collagen fibers area of the scars were compared across all groups. Immunocytochemistry was performed to quantify the collagen type I and III production and fibrillin-1 to determine elastic fibers present in the dermis.

Results

83,3% of the xenografts shed successfully. The mean time for shedding was 35±5.4 days. Statistical differences were found in the total area, thickness and collagen fibers area in the comparison between the groups. Reduction in 10% of total area of the scars was achieved with the treatment in comparison with the

placebo group. An increase in the expression of fibrillin-1 and decrease in collagen I were found when comparing treatment group with the basal group.

Conclusions

Topical application of the inhibitor of TGF-β1 seemed to be promising in an "in vivo" model of human hypertrophic scars implanted in nude mice given that enhancing of scar maturation was found with a decrease in dermal collagen fibers and an increase in elastic fibers. Although increasing knowledge in the pathogenesis of hypertrophic scars has been achieved for many decades, it is clear that there is still a lack of an ideal animal model that would allow understanding this disease in a more holistic approach. Consequently, a more extensive knowledge of the pathogenesis would help to focus the new therapies on modulating the fibrotic disorders pathways.

RESUMEN

Antecedentes

Las cicatrices hipertróficas suponen una de las complicaciones más relevantes en el campo de la cirugía debido a sus implicaciones cosméticas y funcionales. *Transforming Growth Factor-beta* (TGF-β) es uno de los reguladores más representativos responsables de una cicatrización anómala. En estudios previos donde se han utilizando inhibidores de la acción del TGF-β1 en enfermedades caracterizadas por exceso de fibrosis han demostrado efectos prometedores. El objetivo del presente estudio es analizar el efecto de la administración tópica del péptido inhibidor del receptor tipo III del TGF-β1 (P144®) sobre cicatrices humanas implantadas en ratones atímicos.

Material y métodos

Se tomaron muestras de cicatrices hipertróficas de 30 pacientes y se implantaron en 60 ratones atímicos. Los ratones se dividieron en dos grupos, grupo A (placebo) y grupo B (tratamiento). El grupo C (basal) fue constituido por las muestras de las cicatrices hipertróficas sin implantar. Una vez que prendieron todas las cicatrices sobre los ratones se procedió a la aplicación diaria de un lipogel que contenía o bien un vehículo o bien P144 sobre todas las muestras durante 15 días consecutivos. Después de finalizado el tratamiento, los animales fueron sacrificados y se extrajeron dichas cicatrices hipertróficas. El área total, el grosor y el área ocupada por fibras de colágeno fueron cuantificados para su comparación entre los distintos grupos. El estudio inmunohistoquímico se realizó para determinar la expresión de colágeno I, colágeno III y fibrilina-1.

Resultados

El 83.3% de los xenoinjertos prendieron totalmente. El tiempo medio para el prendimiento fue de 35±5.4 días. Diferencias estadísticamente significativas se encontraron en el área total, grosor de la cicatriz y área ocupada por fibras de

colágeno en la comparación entre los tres grupos. La reducción en el área total secundario al tratamiento fue de un 10% en comparación con el grupo placebo. Un aumento significativo en la expresión de fibrillin-1 y una disminución de colágeno I fue hallado en el grupo que recibió el tratamiento comparado con el grupo basal.

Conclusiones

La aplicación tópica del péptido inhibidor del TGF-β1 en el modelo "in vivo" de cicatrices hipertróficas implantadas en ratones atímicos ha demostrado revertir la cicatrización a su curso normal con la consiguiente disminución del contenido en colágeno y reordenamiento de las fibras elásticas. No cabe duda de que existe un progresivo conocimiento en la fisiopatología de la cicatrización hipertrófica, sin embargo aún sigue siendo necesario la búsqueda de un modelo animal que permita estudiar este desorden de forma global. Con ello, alcanzaremos un conocimiento aún más extenso que facilitara nuevos tratamientos enfocados en bloquear las vías reguladoras presentes en estos desórdenes fibróticos.

INTRODUCTION

1. Clinical relevancy of the hypertrophic scars

Peacock[1] described hypertrophic scars as typically present as elevated, red, itchy, firm lesions that stay within the confines of the original lesion. They usually develop within the first one to three months after injury and can typically decrease in size with time. Hypertrophic scars pose a clinically relevant problem as it can be cosmetically disfiguring and functionally debilitating[2,3]. The frequency of encountering excessive scarring after injury, trauma or surgery was 15%[4]. This number increased in burn injuries. The high frequency of hypertrophic scarring found in the latter patients is not insignificant, ranging from 32 to 72%[5]. From many studies of burn injured survivors, hypertrophic scarring seems to be the aspect that affects their quality of life more deeply, and that in turn can lead to

lowered self-esteem and social isolation. Risk factors for hypertrophic scar development are numerous and several studies state their cause-effect relation. The suture material, wound closure tecniques[6], incision site[7], differences in ethnic[8] or genetic predisposition[9] and deep dermal injuries were associated with higher percentage of hypertrophic scarring. In fact, in the study of Hassan et al.[10] dealing with the relationship between healing time and formation of hypertrophic scars in burn injuries, the only factor statistically significant in predicting healing time was depth of burn. Consequently, deeper burn injuries were more susceptible to develop hypertrophic scarring. This result is supported by another study where the authors demonstrated that the contribution to the fibrosis formation of those fibroblasts present in the deep dermis was substantially more relevant than those present in the superficial dermis layer[11]. Moreover, the former have some similarities to those fibroblasts found in hypertrophic scars. There is still lack of knowledge about the basis of this pathology in spite of the high number of studies regarding the wound healing's mechanobiology. In the context of growing aesthetic awareness, a rising number of patients feel disappointed with their scars having a great impact on their quality of life and consequently should encourage physicians to seek effective treatment and prevention. Nowadays the outcome of the surgeries strongly depends on the final appearance of the scars, especially in

the field of Plastic Surgery where the patients' expectation is higher than that of the average population[12].

1.1. Main differences between hypertrophic scar and keloid scar

Keloid scar is a result of abnormal wound healing process with an unexplored etiology. It is a benign dermal fibroproliferative tumor characterized by an excessive accumulation of extracellular matrix, leading in particular to an overabundance of collagen formation[13]. Regarding time of appearance, hypertrophic scar results from a prolonged healing process, with excessive signaling of pro-inflammatory regulators and usually appears after several months, whereas keloid scars can appear spontaneously after more than 12 months from the injury.

There is a lack of knowledge regarding the etiology of keloid scars. It seems that familiar predisposition is a crucial factor[14]. Other related factors are the hormone influence (higher incidence in puberty, pregnancy), age (10-30 years old are at higher risk) and ethnic (Hispanic, Afro-American or Asian are more prone)[15]. So far, none of the above has contributed significantly to the understanding of the pathogenesis.

Commonly, keloid scars are characterized by its extension beyond the confines of the original scar invading the normal surrounding tissue[16] while hypertrophic scars are confined in the original wound. The latter usually regress over time and keloid scars do not[17].

Histologically, keloid scars are provided with thick collagen bundles and protomyofibroblasts instead of myofibroblast as occurs in hypertrophic scars. Opposite to myofibroblasts, there is absence of α-SMA in protomyofibroblasts, and thus, an inability to develop enough forces to contract the scar. Besides, they can produce large amounts of extracellular matrix proteins[18]. Similar to hypertrophic scars, the TGF-β superfamily molecules may contribute to the excessive fibrotic tissue production and to keloid invasive behavior[19].

The clinical course and physical appearance define keloids and hypertrophic scars as different entities, however, they are often confused because of an apparent lack of morphological differences. In fact, the existing strategies for the management of hypertrophic scars and keloids are mainly similar. With a progressively deeper knowledge about their histological differences, in the future, the therapeutic approaches might succeed at developing specific treatment for these different types of pathological scars.

2. Current knowledge in wound healing process

Many authors have conducted excellent studies on wound healing process regulation in the past decades. Although the current knowledge is deep it is not broad enough since there are still many questions without an answer.

Wound healing is a complex and multifactorial process, which proceeds through several overlapping dynamic phases although the true mechanism is not well established yet[20]. Depending on the authors, wound healing proceeds through four or five phases[21] and it involves an interlinked series of molecular and cellular mechanisms. During this dynamic, interactive and complex process, abnormal response of some of these components may lead to abnormally excessive fibrosis, resulting in hypertrophic or keloid scarring or by contrast, to a chronic ulcer.

2.1. Inflammatory phase

Inflammatory phase occurs immediately after the injury and enables the homeostasis and prevention of the infection mediated by induction of cell proliferation and recruitment of platelets, neutrophils and macrophages. The goal is the formation of blood clot and a provisional matrix to fill the tissue defect. Platelets present in the wound start to release pro-inflammatory cytokines such as

platelet-derived growth factor (PDGF), TGF-β, fibroblast growth factor (FGF)[22]. These growth factors that regulate cell migration and proliferation, at the same time may perpetuate the cascade of cells recruitment and increase the production of extracellular matrix proteins by fibroblasts.

2.2. Proliferative phase

After the first 2 to 3 days after injury proliferative phase starts, its main objective being the creation of new capillaries to nourish the neo-tissue and the cells present in the wound bed, enhancing the production of granulation tissue. Fibroblasts play a key role in this phase, not only because of the release of pro-inflammatory cytokines but also promoting the wound contraction mediated by their differentiation to myofibroblasts[11]. The latter are provided with contractile properties due to α-SMA or stress fibers[23] and have an increase in cytokines and extracellular matrix proteins production and deposition. TGF-β is one of the key cytokines regulating the fibroblasts differentiation to myofibroblasts that is typically enhanced in hypertrophic scarring. Both fibroblasts and myofibroblasts synthesize and promote deposition of extracellular matrix components that replace the provisional matrix.

2.3 Remodeling phase

The third phase involves the remodeling of the tissue and reepithelization resulting in the scar formation at the end. This process may prevail one year after injury. Proteolytic enzymes, matrix metalloproteinases will mediate this phase, with the replacement of collagen type III to collagen type I. The collagens comprise the main structural element of the extracellular matrix, forming a relaxed network of cross-linked long chain fibers to give integrity to the skin[24]. There are twelve types of collagens[25], but only two of them play a key role in wound repair mechanisms, collagen I and III. In the early proliferative phase, expression of collagen III is prominent but during the later stages of the proliferative phase and at the beginning of the remodeling phase collagen I fibers replace collagen III fibers since collagen I fibers are stronger. Several studies in keloid scars stated that the ratio of Collagen I/III was 17:1 whereas in normal scars was 3:1 or 6:1[26]. The ratio of collagen I/III was not determined for hypertrophic scars. The synthesis of collagen type I and III has shown to be up-regulated in both keloid and hypertrophic scarring.

Few studies include the changes in elastic fibers deposition in hypertrophic scarring processes. They usually reappear in the late remodeling phase associated

with progressive scar maturation. Although it is clear that elastic fibers are one of the major extracellular matrix components, there is debate about their significance in pathological conditions[27,28]. Elastic fibers are present in large amounts in normal skin and that endows tissues to passively recoil without energy input. The two major structural components of elastic fibers are fibrillin-rich microfibrils surrounding a central core of amorphous hydrophobic cross-linked elastin[29]. Clinical improvements may occur concomitantly with the rearrangement of elastic system components.

2.4. Resolution phase

In the resolution phase, the cell density normalizes through apoptosis of the vascular cells and myofibroblasts[30]. In normal wound healing process this phase leads to the scar formation. Overexpression of some of the regulators or prolonged inflammatory response may lead to hypertrophic scar formation whereas deficient wound healing can lead to the formation of chronic ulcers.

3. Main factors involved in the hypertrophic scarring

Several growth factors and cytokines have been studied for their role in wound healing[31]. TABLE 1 represents the main factors involved in the wound

healing regulation, explaining the mechanism that results in profibrotic processes. Cells, growth factors, hormones, proteins and genes play a key role to restore the tissue defect after the injury. None of these factors stands alone in the wound healing process. Through either paracrine or autocrine effect they will affect the action and the signaling of each other. This process is dynamic and the final result is based on the cross talk between different components. Briefly, these elements regulate the production of cytokines, either pro-inflammatory, such as IL4, IL5, IL 10, IL 13, IL21, TGF-β, PDGF, CTGF, IFN II or anti-inflammatory, such as IL12, IFNα, IFNγ, bFGF; promote the production, deposition and degradation of extracellular matrix components and cell differentiation and apoptosis.

Among all these elements, many studies stated that TGF-β plays a critical role in the development of skin fibrotic diseases[32-34]. This cytokine is the closest and most representative cytokine that promote fibrosis and scarring in different tissues since its identification and purification in 1983[35,36]. Its role is crucial in cell differentiation, development and homeostasis[37].

Table 1. Main factors involved in wound healing regulation

Legends:

proCol: pro-collagen

MMP: matrix metalloproteinases

TIMP: tissue inhibitor of metalloproteinases

NO: nitric oxide

PDGF: platelet-derived growth factor

IGF: insulin growth factor

bFGF: basic fibroblast growth factor

SLRP: small leucine-rich proteoglycan

VEGF: vascular endothelial growth factor

HGF: hepatic growth factor

EGF: endothelial growth factor

BM-MSC: bone marrow mesenchymal stem cells

CTGF: connective tissue growth factor

SMA: smooth muscle actin

SDF: stromal-cell derived factor

PAI: plasminogen activator inhibitor

CBPG: collagen binding proteoglycans

LH-2b: lysyl hydroxylase

TRP: TRAP-1 like protein

Cav: caveolin

TLR: toll-like receptors

PPAR: peroxisome proliferation activated receptor

BMP: bone morphogenetic protein

PTEN: phosphatase and tensin homologue deleted on Cr. 10

Element	Nature	Origin	Mechanism	Effect
Th-2[32]	T-cells	CD4+	Production of IL4, IL5, IL10, IL13, IL21, gen expression of proCol I, III, arginase-1, lysyl oxidase, MMP-2 and MMP-9 and TIMP-1	Profibrotic
Th-1[32]	T-cells	CD4+	Production of IL12, IFNγ, Activation of collagenase by activation of NO synthase	Antifibrotic
Fibroblast[38]	Cell		Production of IL6, IL10, TNFα, TGF-β, PDGF, deposition of fibronectin, colI, col III, decrease collagenase activity, production of IL1, TGF-β, IGF, bFGF	Profibrotic
Myofibroblast[11]	Cell		Increased activity of fibroblast	Profibrotic
Fibrocyte[39]	Cell		Production of TGF-β1, enhance Col I production, proteoglycan, versican, decrease production of SLRP	Profibrotic
Macrophages[32]	Cell		Activation by Th1: Production of IL1a, IL1b, IL6, TNFα, TGF-β, PDGF, FGF2 and IGF-1	Profibrotic
			Activation by Th2: Production of anti-inflammatory cytokines	Antifibrotic
Platelet[22]	Cell		Recruitment of inflammatory cells	Profibrotic
			Production of PDGF, TGF-β, FGF2, VEGF, HGF, IGF, EGF.	
Mast cell[22]	Cell		Production of IL4, TGF-β1, TNFα, Histamine: enhance collagen production by fibroblast "in vivo"	Profibrotic
BM-MSC[40]	Cells		Enhance deep dermal fibroblasts activity	Profibrotic

Element	Nature	Origin	Mechanism	Effect
TGF-β[33]	Growth factor	Platelets, fibroblasts	Promotes collagen synthesis, induce fibroblasts differentiation to myofibroblasts, increase the ECM	Profibrotic
PDGF[32]	Growth factor	Platelets	Fibroblast proliferation and ECM secretion, fibroblast differentiation to myofibroblast	Wound healing, profibrotic
VEGF[22]	Growth factor		Disbalance leads to hypertrophic scarring	Profibrotic
bFGF[38]	Growth factor		Decrease production of Col I	Antifibrotic
FGF[41]	Growth factor		Increase the metabolism, improve wound healing, regulation of the inflammation response	Wound healing
CTGF[42]	Growth factor		Enhance synthesis of fibronectin, col I and α-SMA	Profibrotic
IFN α[32]	Cytokine	Leukocyte, fibroblast	Increase collagenase activity and inhibition of collagenase inhibitor	Antifibrotic
IFN II[32]	Cytokine	T-Cell	Inhibition of collagen synthesis	Profibrotic
SDF-1[43]	CXCchemokine		Chemoattractant for lymphocytes, monocytes and fibrocytes, enhance angiogenesis	Profibrotic
Angio- I[22]	Protein-hormone		Production of TGF-β, enhance ECM synthesis, fibroblast differentiation to myofibroblast, Keratinocyte migration	Profibrotic
Angio- II[22]	Protein-hormone		Increase antifibrotic actions	Antifibrotic
Decorin[44]	SLRP		Decrease collagen fibril formation and cross-linking, Decrease expression of α-SMA and PAI-1 "in vitro"	Antifibrotic

Element	Nature	Origin	Mechanism	Effect
CBPG[45]	Proteoglycan	Fibroblast	Inhibition of TGF-β1 "in vivo" and "in vitro"	Antifibrotic
Versican[46]	Proteoglycan	Fibroblast	Enhance adhesion, migration and proliferation	Profibrotic
Periostin[44]	Protein		Fibrillogenesis of Col I	Profibrotic
PAI-1[22]	Protein		Production of IL4, TGF-β, PDGF	Profibrotic
LH-2b[11]	Enzyme		Collagen fibril cross-linking	Profibrotic
TRP[47]	Protein		Enhance col III synthesis	Profibrotic
Thy-1 or CD90[45]	Protein		Decrease fibroblast differentiation to myofibroblast	Antifibrotic
Cav-1[48]	Protein		Decrease activity of TGF-β	Antifibrotic
TLR[49]	Protein		Production of cytokines, chemokines	Profibrotic
PPAR-γ[50]	Protein		Inhibition of TGF-β and CTGF	Antifibrotic
BMP-7[51]	Protein	TGF-β	Reverse TGF-β profibrotic effect	Profibrotic
Wnt genes[52]	Gen	Wnt-b-catenin	Decrease expression of α-SMA, col I, col III	Antifibrotic
PTEN[53]	Gen		Inhibition of collagen synthesis	Antifibrotic

TGF-β is a 25 kDa homodimeric protein identified in mammalian cells[54]. TGF- β is secreted by numerous cells, mainly by platelets, activated T-cells, macrophages, neutrophils and bone marrow. There are three isoforms: TGF-β1, TGF-β2 and TGF-β3[20]. Among the three types of cytokines, TGF-β1 and TGF-β2 play the main fibrogenic role whereas TGF-β3 inhibits the effect[34,41]. TGF-β1 promotes mainly three functions: collagen synthesis and deposition by stimulating the fibroblasts, deposition of other extracellular matrix proteins and the induction of fibroblasts to myofibroblasts differentiation, the latter provided with α-SMA[52]. TGF-β1 signaling pathways function through the TGF-β type I and type II transmembrane serine/threonine protein kinase receptors[20]. Activation of the receptor complex occurs when type II receptor transphosphorylates the glycine-serine domain of type I. Then the activated type I kinase associates transiently with a transmembrane receptor, Smad2/Smad3. When they are phosphorylated, this complex is bind to Smad4 and then enters the nucleus, where it activates a target gene transcription such as α-SMA expression and collagen I synthesis[47,55]. According to previous studies[32], a third receptor exists, receptor type III and its ligands activate receptor I and II by phosphorylation and the inhibition of the latter suppresses the action of these two receptors. This peptide has been isolated and can be tested as a potential new therapy for fibrotic diseases.

4. Currently available therapies for hypertrophic scars

For many decades, different modalities of prevention and treatment of hypertrophic scars have been used. Unfortunately, none of them has reached total remission of these scars. The most popular therapies currently used are silicone based-products, steroids and laser therapy. Many other treatments have emerged based on the knowledge of the mechanism of abnormal healing and they are shown in detail in Table 2.

A variety of mechanisms have been used in the attempt to achieve clinical improvement of the hypertrophic scars. The following represents the main ways to reduce the fibrosis: induction of tissue hypoxia to provoke vascular damage (cryotherapy[56], intense pulsed light[57]); enhancing cell apoptosis (pulsed dye laser[58]), inhibition of cell proliferation (steroids[59], radiotherapy[60], 5-Fluorouracile[61]); rearrangement of misbalance between pro-fibrotic and anti-fibrotic regulators (IFN α2b[62], avotermin[63]); and increase collagen breakdown (imiquimod[64], pressure garments[27], steroids[65]) as some examples of the pathways used to reduce the fibrosis. Nowadays polytherapy is becoming more popular because gives the chance to combine different mechanisms and theoretically

Table 2: Current therapies for hypertrophic scars.

Legend:

LE: level of evidence

RF: radiofrequency

VSS: Vancouver scar scale

RT: radiotherapy

PG: prostaglandins

BTXA: botulinum toxin type A

ASA: acetylsalicylic acid

BBB: blood-brain barrier

HMME: hematoporphyrin monomethyl eter-mediated

UV: ultraviolet

LE	Therapy	Application	Mechanism	Effect	Duration	Side Effects
4	Surgery[66]	Mechanical	Excision	Reduction		Recurrence
4	Cryotherapy[56]	Mechanical	Vascular damage, fibroblast apoptosis	Cure 32-74%		Hypopigmentation, pain
4	Silicon, sheeting, fluid gel[67,68]	Topical	Increased oxygen delivery, hydration of stratum corneum, skin temperature and reduced tissue turgor. Decrease TGF-β2 and fibroblasts	Soften, increase elasticity, better appearance		
4	Triamcinolone[65]	Injection	Inhibition of fibroblast, increase collagenase, apoptosis, decrease mast cells	Flattening	2-4 w	Pain, skin atrophy, hypopigmentation
4	Triamcinolone[59]	ablative RF		Complete resolution	4 sessions.	
4	Steroids ointment[69]	Injection and topical		Lower recurrence	1/2w till 5 times 2/d for 6 m	
4	Cryosurgery and steroid[70]	Injection		Volume reduction	4 m	
4	Cyanocrylates[71]	Topical		Reduced elevation		
4	Allium cepa-allantoin-pentaglycan gel[72]	Topical	Decrease angiogenesis		2/d for 6 m	
4	Onion extract gel,hydrocortisone,silicone, vitamin E[73]	Topical		Prevention		Erythema and pigmentation
2	Onion extract in silicone derivative gel[74]	Topical		Prevention		
3	Pressure garment[27]	Topical	Increase MMP-9, reduce collagen synthesis.	Improve hardness,color thickness		Expensive,skin breakdown,low adherence

LE	Therapy	Application	Mechanism	Effect	Duration	Side effects
4	Intense pulsed light[57]	Laser	Tissue hypoxia and collagenolysis	Improve height, Erythema, hardness		Temporary erythema, burning
4	Nonablative fractional laser ± Pulsed dye laser[75]	Laser	Selective thermolysis	Better thickness, pigmentation		
4	Ablative fractional photothermolysis[76]	Laser	Tissue vaporization			
2	Nonablative fractional resurfacing[77]	Laser	Tissue coagulation	Pliability		
4	532-nm potassium titanyl phosphate[78]	Laser		Better appearance, pigmentation	1 session/m 4 m	
4	Laser therapy[79]	Laser	Acute inflammation, increase MMP Increase cell proliferation Increase collagen I collagen III and elastin.	Better VSS		
4	Continuous-wave[80]	Laser				
4	Pulsed dye lasers[58]	Laser	Fibroblasts apoptosis			
4	Q-switching[58]	Laser		Decrease erythema and pruritus		Erythema,purpura, hyperpigmentation hypopigmentation
4	Fractional CO2 ablative[81]	Laser	Photothermolysis			
4	Laser+triamcinolona[80]	Laser, injection		Improve thickness	12 m	

LE	Therapy	Application	Mechanism	Effect	Duration	Side effects
4	RT (15-20Gy)[60]	Radiation	DNA ionization	35-45% cure rate	5 or 6 sessions	Malignancy risk, pigmentation, itching,bone marrow supression, ulcers
4	32P-patch contact brachyradiotherapy[82]	Radiation		Decrease size		
4	5FU[Y61]	Topical	Inhibition thymidine synthase	Reduction size	Weekly/12w	
4	imiquimod 5% cream immune-response modifier[64]	Topical	Collagen breakdown, reduction collagen synthesis		8w	Irritation, hyperpigmentation
4	Tranilast[83]		Inhibition histamine and PG H1 receptor antagonist, inhibition collagen	Improvement of itching		
4	Putrescine (fibrostat)[84]		Inhibition of transglutaminasa	Clinical improvement	4w	
3	IFN-α2b[62]	Injection	Induction of scar remodeling and maturation, inhibition TGF-β1, myofibroblast apoptosis and decreased angiogenesis and CXCR4	Normalizing TGF-β Volume reduction		Myalgias, low-grade fever, fatigue
4	Mitocycin C[85]	Topical injection	Inhibition DNA replication	VSS improvement		Intralesional application can aggravate
4	Bleomycin[86,87]		Decrease collagen synthesis	Flatenning		Hyperpigmentation,dermal atrophy
2	Verapamil[88]	Injection	Increase decorin, alters fibroblast shape, induce collagenase, reduce extracellular matrix proteins.	Height, width reduced		
3	Avotermin, TGF-b3[63]			Prevention		
4	BTXA[¢] 35U/ml[89,90]	Injection	Reduction of TGF-β1 secretion, inhibition of the growth and differentiation of the fibroblast in vitro	Prevention of widening	1m, 3m, 9m	

Model	Therapy	Application	Mechanism	Effect	Duration	Side effects
Nude mice	Antisense oligodeoxy Nucleotide (ASODN)[91]	Injection	Inhibition synthesis of collagen I in vitro	Flattened dermis, less collagen I		
Nude mice	Inhibior of chemokines CXCR4[43]	Injection	Induce collagen fibers, Reducing macrophages and myofibroblasts			
Rabbit ear	Zinc oxide[92]	Topical		Decrease hypertrophy	1/d, 3w to 6 w	
Rabbit ear	Basic FGF[93]	Topical	Inhibition collagen synthesis, collagenolysis		3 /d, 1, 2 and 3 m	
HTS Fibroblast	Decorin[94]	Injection	Bind TGF-β1 in renal fibrosis.			
Rabbit ear	Recombinant stratifin or ASA in carboxymethyl cellulose gel[95]	Topical	Collagenolysis		23 d	
Rabbit ear	Indoleamine 2,3 ddioxygenase[96]	Topical	Increase MMP-1 and 3, suppresses of collagen I and Fibronectin			Passes BBB
Rabbit ear	Angiotensina[97]	Oral	Decreased fibroblasts and vascularization Inhibition proliferation and collagen synthesis			
Rabbit ear	Hepatocyte growth factor[98]	Injection	Inhibition fibrosis	Decrease elevation		
Rabbit ear	Tacrolimus[99]	Injections	Inhibition of proliferation	Decreased thickness		
Rabbit ear	Trichostatin[100]	Injection	Inhibition collagen synthesis and fibronectin	Decreased elevation		
Rabbit ear	Endostar[101]	Injection	Collagenolysis, inhibition of proliferation		1/w, 3w	
HTS Fibroblast	5-aminolevulinic acid photodynamic[102]	Laser	Apoptosis			
HTS Fibroblast	HMME photodynamic[103]	Laser	Apoptosis			
Rabbit ear	High-dose UV[104]	UV light		Reduction volume	7-14 d	

may be more effective to reduce the hypertrophy.

The main outcome to test different therapies is the improvement of the clinical appearance of the scar. The goal is to obtain a normal, thin and flat scar. Not only the reduction in volume and elevation but also improvement of color, softness and elasticity as well as decrease in pruritus are crucial features that measure the effectiveness of any treatment.

4.1. Evidence-based medicine therapies

Silicone-based products, laser therapy and pressure garments were the therapies susceptible of systematic reviews since they represent the best benefits vs. risk relation in treatment for hypertrophic scar. They are commonly known as first-line treatments. However, to date none of these systematic reviews has demonstrated the effectiveness of any of them.

O'Brien et al.[105] conducted a systematic review of the efficacy of silicone gel sheeting using all randomized controlled trials available. Due to the poor quality of the previous design for the research, the improvements in scar thickness and scar color were highly susceptible to bias and it was not possible to state a definite conclusion.

Three systematic reviews studied the effect of intense pulsed light therapy[106], pulsed dye layer[107] and laser therapy[79] in the treatment of hypertrophic and keloid scars. Laser proved to be safe in hypertrophic scar management. Although the authors concluded that more standardized methods of treatment and outcome assessment were recommended in order to prove its effectiveness.

In the systematic review conducted by Anzarut et al.[108] pressure garment therapy did not alter the global appearance of the scar while the potential morbidity and cost were significant. The results were not conclusive and they suggested that further studies be done to examine the effectiveness, risks and costs of pressure garment therapy.

In the light of these previous studies, further studies are needed to assess the real effectiveness of different therapies against hypertrophic scars. Well-designed trials, with established outcomes, with consensus reached in the way to measure these outcomes and during a long period of time should be encouraged to reach higher levels of evidence in this issue.

4.2. Prevention of hypertrophic scars formation

The therapies that can prevent the formation of hypertrophic scars are emerging and the results seem to be promising. Usually the agents are less aggressive, with less morbidity and very few side effects. Their application is commonly less harmful and the treatment adherence is usually high.

A combination of onion extract in silicone derivative gel[74] showed promising results in prevention of hypertrophic scars but not for keloid scars in pediatric patients. This gel is applied topically with no side effects.

Microporous hypoallergenic paper tape has demonstrated its efficacy in prevention of hypertrophic scarring in a randomized controlled trial[109] by means of eliminating stretching forces and controlling wound edges tension. The authors recommend to start using it on scars within the two weeks following surgery and for a minimum of three months.

In a triple-double blind, placebo –controlled study, So et al[110] demonstrated that Avotermin, an analogous of TGF-b3, was able to prevent excessive scar formation after full thickness skin incision. Although its use has not yet been translated to clinical setting, the results seem to be promising.

4.3. Emergent therapies

To date, as it has been demonstrated above, none of the currently used treatments are fully effective in achieving clinical improvement with favorable long-term results. Seeking for the ideal treatment is still a challenge. New therapies have shown good results either “in vitro” or “in vivo”.

Many of them reduce fibrotic process by inhibition of collagen synthesis, such as anti-Co 1 antisense oligodeoxynucleotide[111] (ASODN), and its effect has been demonstrated in cultured fibroblasts; basic FGF[93] also induces collagenolysis; and so does histone deacetylase inhibitor[100].

Decorin[112] is a natural antagonist of TGF-β fibrotic activity and its effectiveness was tested in renal fibrosis. Indoleamine 2.3 dioxygenase[96] is available for topical application and can increase the MMP-1 and 3 activity, enhancing the collagen fibers breakdown.

Some agents can inhibit cell proliferation such as tacrolimus[99] and angiotensin[97] whereas others can induce cell apoptosis such as 5 aminolevulinic acid photodynamic laser[102] and high-dose ultraviolet light[104].

The results shown in animal models or in vitro were promising for many of these emergent agents. Further studies in order to increase the knowledge of their efficacy and safety are encouraged before the application in patients.

4.4. TGF-β1 blockers

Among the cytokines presented in the wound healing process, some of them show TGF-β1 antagonist effect. The majority of them decrease the TGF-β1 signaling by blocking its effector receptors or its effect in the different targets. Smad 7 belongs to Smad proteins responsible of intracellular effect of TGF-β1 signaling. Smad 7 is the unique negative feedback regulator of TGF-β1[41]. IFNa2b down-regulates the expression of TGF-β1, promoting myofibroblast apoptosis and decreasing the angiogenesis mediated by vascular endothelial growth factor (VEGF). A trial in burned patients showed its effectiveness in subcutaneous administration. EGF decreases the collagen type III synthesis and the levels of α-SMA regulated by TGF-β1[41]. In addition, an increased expression of decorin, a small leucine rich proteoglycan, may decrease TGF-β1 fibrotic activity[112,113]. TNFα inhibits extracellular matrix synthesis, activates matrix metalloproteinases and inhibits the α-SMA expression and subsequent myofibroblast differentiation in human dermal fibroblast.

Botulinum toxin A reduces the TGF-β1 secretion and can inhibit the fibroblast growth in human hypertrophic scars[114,115]. Putrescine or 1,4 diaminobutane induces fibroblast apoptosis and decrease the fibrotic effect of TGF-β1 by inhibition of tissue transglutaminase[32,84]. Tetrandine[116] produces an up-regulation of Smad7 and consequently inhibits the effect of TGF-β1 as it is described above.

Regarding the inhibition of TGF-β1 receptor, several peptide inhibitors have been described. Querceptin[117] can inhibit TGF-β1 receptor type I and II, Smad2/3 system, in keloid fibroblasts. Several studies have shown the effect of an inhibitor of TGF-β1 (P144®; Digna Biotech. Spain) in reducing the fibrotic condition of different tissues, such as myocardial fibrosis[118]; liver fibrosis[119] with intraperitoneal administration of P144 in rats; scleroderma[120] using topical administration in human implanted skin samples in C3H mice and nerve regeneration[121], where the application of P144 was performed after sciatic nerve section and coaptation in rats. All the aforementioned processes present pathological fibrosis and these specific features were shared with the hypertrophic scarring. Although no studies have been conducted to date to test the effect of P144 in the treatment of hypertrophic scars, it seems to be reasonable to study its

effects. Therapies that enable to modulate the TGF-β1 effect appear to be the most promising, due to the key role of this growth factor in hypertrophic scarring.

5. Described animal models for the study of hypertrophic scarring

Reproducing hypertrophic scars in animals is still a challenge since this kind of fibrotic scarring only occurs in mankind[122]. Moreover, because hypertrophic scarring is a process that develops slowly, it can be difficult to obtain a long-term hypertrophic scar in an animal model that resembles human scars. Although no ideal model has been described yet, there is no doubt that animal models for human hypertrophic scars are crucial for performing systematic studies to understand the pathogenesis of this disease and for testing new therapies.

Many ways to create hypertrophic scars in animals have been described. The animals used could be large, such as white pigs or rabbits and small, like murine models. The later are the most popular and the most frequently used because of their availability and their easy caring and housing.

Many techniques have been described to reproduce a hypertrophic scar that resembles a human scar. Briefly they can be produced by: (1) inducing excessive fibrosis by injury, such as burning in order to create a deep dermal partial thickness in large white pigs[123] or producing surgical scars on red duroc pigs[124,125]; (2) using hypertrophic scarring predispose areas such as rabbit ears[89], by means of creating a full thickness ulcer; and (3) by direct implantation of human tissue samples in athymic mice. Numerous models have been used with nude mice and this is the model chosen for the current study. They will be discussed in detail below.

Congenitally athymic nude mice (nu/nu) represent a model for cell-mediated immunologic deficiency[126]. These mice possess a vestigial thymus, which is incapable of producing mature T-cells leading to deficiency of T-cell dependent functions and an absence of delayed hypersensitivity. On the other hand, they preserve spontaneous autoimmunity and B-cell hyperactivity associated with T-cell deficiency. For many decades these animals have been used for studying pathogenesis of congenital cellular immunology disorders in human kind, tumor pathogenesis and other diseases.

Introduction

The use of nude mice for keloid scars pathogenesis study was popularized by Shetlar[127] and nowadays it is world-wide used for xenografts transplantation. Implantation of human hypertrophic scars reproduces an identical scar on a mouse and allows to test different therapies by topical application[120], transdermal patch sheets[37], selective photermolysis[128] or subcutaneous injection[91,129-131] that is the most frequently used way. The level of glycosaminoglycan on the implanted hypertrophic scars can last till day 60[132] or 80[133] and then the level of these proteins starts to decrease. For this reason, this model is not suitable for the studies that require a longer period of time.

Other models tried to create a hypertrophic scar by implantation of human skin graft, either full or split thickness skin on the nude mice. After the skin grafts were shed successfully, it is recommended to wait for several weeks to achieve the status of hypertrophic scar. The time needed to obtain a histological hypertrophic scar varies according to different authors from 30 days to 216 days[134-136]. In the study performed by Wang et al.[135] they stated that the split thickness skin graft produced more fibrosis than full thickness skin graft after two months.

The injection of genetically modified human fibroblasts with overexpression of TGF-β1[137] was another way to create hypertrophic scar in these

animals. The resulting scars present histological and morphological features of hypertrophic scars. Either using skin grafts of normal human skin or implantation of genetically modified fibroblasts, the response of each tissue sample in these mice might not be homogenous due to basal differences. The resulting scars should be analyzed and the differences should be addressed before starting the study.

The most suitable animal model to compare the effect of a new therapy seems to be the implantation of the same human hypertrophic scar in two mice. For this reason we chose this method to develop our study. This method allows treating pairs of mice with the same hypertrophic scar, one with placebo and one with treatment. The implantation of the same scar enables to have better control of the differences appearing after the grafts shedding process. In addition, keeping a sample of each tissue without implantation as basal group helps to distinguish the changes owing to treatment from the changes due to implantation itself. The scars are commonly ready for study after a short time; the shedding process ends after 2 to 3 weeks after the implantation.

6. Current study characteristics

We used an "in vivo" model of hypertrophic scars to study the effect of P144 in human hypertrophic scars with the aim to recreate a scenario that would resemble the clinical setting as much as possible. The chosen model was the bilaterally implanted human hypertrophic scar in nude mice. After the shedding process ends, the hypertrophic scars were explanted and underwent histological, immunohistochemistral and morphometric analysis in order to study the collagen and elastic fibers. Macroscopic improvement features represent the main goal given that they have clinical impact. Total area and thickness of the scars were quantified and statistical significance was found in the comparison between the groups. A clear trend of collagen I decrease and an increase of fibrillin-1 was found comparing treatment group with placebo group. In the light of the findings obtained in the current study, it seems that topical application of P144 in an "in vivo" model of human hypertrophic scars in nude mice may reduce clinically the total volume of the scars and improves scars' maturation.

HYPOTHESIS AND OBJECTIVES

Hypothesis

The inhibition of TGF-β1 receptor III has an effect in changing either morphological or histological features of human hypertrophic scars implanted in nude mice.

Objectives

1. Measure the macroscopic changes in the hypertrophic scars due to the treatment.
2. Study the impact of the treatment in scar maturation by determining the changes in deposition and density of both collagen fibers and elastic fibers.
3. Establish new prevention actions to avoid hypertrophic scarring.

MATERIAL AND METHODS

1. Obtaining human hypertrophic scars

Protocols to obtain human tissue samples have been approved by the University of Navarra Health Research Ethics Board. All the patients gave verbal informed consent to be enrolled in the current study and this information was written in the medical record of each patient. Correction of the hypertrophic scars in these patients was undergone to improve the mobility of the affected area, in cases of a second surgery in the same area using the same scar to approach or as debulking procedure with the attempt to improve the scars cosmesis. Collecting the resected scars for experimental purpose was secondary to the surgery and none of the enrolled patients were asked to undergo the surgery only for research purpose. The hypertrophic scars were obtained from consecutive patients who

underwent elective surgery for scar resection. A total of 30 patients enrolled in the study. Due to intrinsic differences in epidermal and dermal thickness depending on the location, only scars occurring in the thorax, back or shoulders were included. Epidermis thickness can vary from 0.07 to 0.12 mm whereas dermis may have from 0.66 to 3mm of thick depending on the anatomical area[138]. Only female Caucasian patients were included to avoid ethnic or gender differences. The hypertrophic scars had to be clinically evident for over 6 months since the injury presenting the characteristic features of elevation, hardness and in some cases itching and color change confined in the boundaries of the lesion[1]. According to previous studies, scars reach maximum scores of hypertrophy between 6 to 12 months[139] after their appearance. For this reason, only the scars in this time interval were included in the study.

The scars were enveloped in a sterile gauze with normal saline serum at 4 to 6°C in order to preserve their biological features intact[140]. The implantation of the scars to nude mice was performed within the first 24 hours.

2. Implantation of human hypertrophic scars in nude mice

The current animal study was conducted in accordance to protocols approved by the Institutional Animal Care and Use Committee and according to the European Communities Council Directive (2010/63UE). A sample size recommended to find the mean differences of quantitative variables between two groups with a Type 1 error fixed at the level of 5%, the power of the study kept at 80%, mean difference at 2 and standard deviation at 4 it was 60 animals. Sixty BALB/c-nu/nu T-deficient nude mice (Harlan Laboratories) were assigned in two groups. The body weight ranged between 20-25 g and the age was 4-6 weeks. The animals were placed in individual cages provided with positive pressure to prevent contamination or infection taking into account their susceptibility to these hazards[141]. Under sterile conditions and in a laminar airflow cabinet, each tissue sample was split into three pieces of 1x1 cm in size after removing the excessive subcutaneous fat, as described by Shetlar et al.[133]. Epidermis was preserved intact. Two pieces from the same scar were implanted onto the back of two different mice (one each from group A and group B) and the third piece was used as the basal group and directly fixed in formalin for histological studies (group C). Therefore, comparisons were made between pairs of mice with the same

hypertrophic scar to avoid potential bias. In order to increase the skin grafts take rate, after completing the implantation of the tissue samples in the back of the nude mice, a piece of sterile sponge was sutured above the scar. Group A was to receive placebo and group B would receive P144®. After the shedding period, all the transplanted scars received daily topical application of a lipogel containing either placebo (group A) or P144® (group B) for 2 weeks. The application covered the whole surface of the implanted scar provided with the epidermis and a soft massage was applied to enhance the absorption. The composition of the lipogel was already determined: Dimeticone 350 10%, liquid paraffin 40%, clorocresol 0.1%, cetrimide 0.5%, cetoestearilic alcohol 5%, DMSO 0.2% in distilled water up to 100%. For group B, P144® at 300μg/ml was added to the lipogel.

3. Histological analysis

After 44.6-55.4 days (mean time of 47.8 days) the mice were sacrificed according to the protocol and the whole scar was excised from the back. Biopsy specimens were fixed in 10% formalin for 24 hours and embedded in paraffin. Paraffin sections were cut into 3μm thick slides and subjected to Hematoxylin &

Eosin and Masson´s trichrome staining to study the dermal collagen fibers. There are twelve types of collagen fibers but only types I, II and III belong to interstitial collagens and their fibers are visible under the optic microscope[25]. Collagen I and III are the main types involved in fibrotic disorders. The fibers stain pink with eosine and green with Masson's Trichrome. Collagen type I is present in dermis, bone, tendon and fascia. It is flexible and strong, suitable to endure high tension whereas collagen type III is more abundant in reticular connective tissue, in the vessels' wall, cartilage and vitreous.

3.1. Semi-quantitative measurement of macroscopic changes and collagen fibers

To measure the clinical improvement of the hypertrophic scars, outcomes included macroscopic changes such as total area and thickness of the scars as well as dermal collagen fibers area. Measurement of the total area, collagen fibers area and the thickness of the scars were performed using digital images acquired with a Zeiss AxioCamICc3 camera (Plan-Neofluar objective with 0.50 NA) at 20x magnification with an AxioImager.M1 microscope (Zeiss, Germany). The whole scar including epidermis layer was considered total area for each sample. The quantification of dermal collagen was based on the collagen fibers stained green with Masson's Trichrome. Then, AxioVision software was used to form a mosaic

of the tissue including each picture. Mosaic images were analyzed using an in-house developed plug-in for Fiji (a distribution of Image J) V1.46b. To quantify the scar thickness, an elliptical figure was drawn over the scar image and the shorter axis was taken as its value.

3.2. Morfological assessment of collagen proliferation

The morphology of the epidermis, the presence of skin appendages and the presence of collagen fibers bundles present in superficial and deep dermis were assessed by two independent pathologists using scanned images acquired at 20x magnification. The pathologists were blinded and were not informed about different groups. Comparison was conducted taking the pairs of scars, assessing the changes of the same scar in two different animals after receiving placebo or treatment with P144. Each sample was categorized regarding dermal collagen proliferation as discrete (1), moderate (2), intense (3) and very intense (4).

4. Immunocytochemistry for collagen type I and type III and elastic fibers

The ratio between collagen I/III is altered in excessive fibrotic conditions[26]. The synthesis and deposition of both collagen I and collagen III is enhanced in this pathological condition. In order to determine the distribution and deposition of them separately, immunohistochemical analysis was conducted.

Elastic fibers are formed by fibrillin-rich microfibrils surrounding a central core of amorphous hydrophobic cross-linked elastin[142]. Immunohistochemical staining for fibrillin-1 was performed in order to quantify the amount of elastic fibers present in the scars. Rearrangement of the elastic fibers was associated with maturation of wound healing process and it was usually disturbed in fibrotic conditions[143]. The study of the changes in elastic fibers with inhibition of TGF-β1 activity may bring more knowledge about the impact of normalization of its activity in scar maturation.

Antigen retrieval (AR) was performed for immunohistochemical analysis: heat induced AR (anti-fibrillin 1) was applied for 30 min at 95 ºC in 0,01 M citric acid (pH=6) in a Pascal pressure chamber (S2800, Dako, Glostrup, Denmark). Proteolytic induced AR was performed using a solution of 20μg/ml proteinase K

for 30 min at 37ºC (for anti-collagen I) or 4mg/ml pepsin for 1 hour at 37ºC (for anti-collagen III). Endogenous peroxidase was blocked with 3% H_2O_2 in deionized water for 12 min. and sections were washed in TBS-0.05% Tween 20 (TBS-T). Primary antibodies and their optimal dilutions were: rabbit anti-collagen I (polyclonal, 1:200; 2150-0020, AbD Serotec, Raleigh, NC), mouse anti-collagen III (clone MWD1.1; 1:2000; AM167-5M, BioGenex, Fremont, CA) and mouse anti-fibrillin 1 (clone 11C1.3; 1:100; ab3090, Abcam, Cambridge, UK). Incubations with primary antibodies were performed overnight at 4ºC. After rinsing in TBS-T, secondary EnVision peroxidase-labelled goat anti-rabbit (K4011, Dako) or goat anti-mouse (K4007, Dako) was incubated for 30 min at room temperature. Peroxidase activity was revealed using DAB+ substrate chromogen (Dako) as recommended by the manufacturer. Sections were lightly counterstained with Harris hematoxylin. Finally, slides were dehydrated in graded series of ethanol, cleared in xylene and mounted in Cytoseal XYL (8312-4, ThermoFisher Scientific; Walthman, MA).

To quantitatively assess the expression level of Collagen I, Collagen III and Fibrillin-1, digital images were acquired using an Aperio Scanscope CS2 (Leica Biosystems) at 20x magnification. Images were analyzed using an in-house developed plug-in for Fiji (a distribution of Image J) V1.48v. The total area of the

tissue was segmented automatically to obtain the total area value. Then the images were threshold in order to measure the positive staining area of each marker. The expression level was presented as a ratio of positive area relative to total area. The mean intensity value of the staining was also measured for all threshold areas.

5. Statistical analysis

The obtained data were compared using the Wilcoxon signed rank test or Kruskal-Wallis test, as appropriate. For the subgroups analyses, T-student was applied. All tests were two-tailed. Values of $p< 0.05$ were considered significant. SPSS for Windows was the chosen software for performing the analysis (v.15.0; SPSS Inc., Chicago, IL, USA).

RESULTS

After complexion of the grafts healing process, from the total of 60 grafts implanted in 60 nude mice, 50 were successfully shed (83.33%). Seven mice died probably due to an infection during the post-grafting period. As each scar was implanted in two different mice, when one of these animals died, the other was also sacrificed. Finally, 36 mice (18 pairs) were suitable for testing of P144®'s effect compared with placebo. The mean time of shedding was 35±5.4 days.

1. Morphological features of the implanted scars

Xenografts were shed with the characteristic stiffness, elevation, thickness and confinement to the site of implantation that resemble a human hypertrophic scar. Neovascularization could be seen macroscopically as small vessels reaching the grafts from the surrounding tissue of the host. Fig. 1.

Fig. 1. Macroscopic aspect of a hypertrophic scar in a nude mouse. A. Characteristic hardness and elevation of a human hypertrophic scars after 35 days on the back of a nude mouse. B. After sacrificing the animal, small vessels coming from the surrounding tissue arrived to nourish the hypertrophic scar.

Results

Histologically, the obtained tissue samples resembled human hypertrophic scars. The dermis layer of the implanted scars was thickened and the boundary between papillary and reticular layers of dermis was obscure; collagen fibers were dense, with derangement in the collagen bundles. Irregularly arranged collagen fibers with nodular or whirled shape were found in the dermis. No human skin appendages structures were observed in any of the cases whereas mouse skin adnexal structures were present and preserved their original structure at the boundaries of the xenografts. In some cases, the boundaries of the hypertrophic scar were partially embedded under the mouse skin resulting in an image of mouse skin beneath the hypertrophic scar. The presence of these images depended on where the paraffin section was placed. This was a phenomenon that occurred at the boundaries of the implanted scar but not in the central part. Full thickness scar with intact human epidermis was present in all samples allowing topical application of P144 in all shed specimens. In five cases belonging to the placebo group host vs. graft reaction was found in the specimens secondary to surgical sutures. Fig. 2.

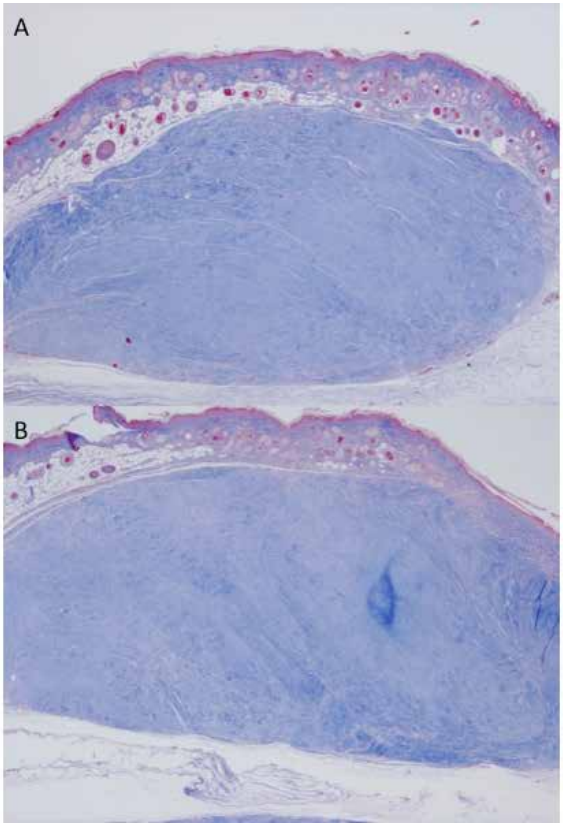

Fig. 2. Morphology of human hypertrophic scar after transplantation to a nude mouse. A. Dermal collagen proliferation with engrossed and nodular collagen bundles. The scar is covered by normal mouse skin. B. Same scar from another section. In this case human epidermis can be seen intact above the scar.

2. Effect of the treatment in macroscopic features of the hypertrophic scars

Table 1 shows the results of total area and thickness of the scars obtained with the Wilcoxon signed rank test for the three groups comparison. Statistical significance was found.

Table 1. Comparison between the groups in total area and thickness

Variable	Group	N	Mean	Std. Deviation	*p* value
Total area (cm^2)	Basal	18	3.6	1.97	
	Placebo	18	2.02	1.10	.005
	P144	18	1.68	1.01	
Thickness (cm)	Basal	18	1.48	0.44	
	Placebo	18	0.99	0.35	.000
	P144	18	0.88	0.36	

In the subgroups comparison, statistical differences were found between basal vs. placebo (p=.006) and basal vs. P144 (p=.005) in total area. Similar results were found in thickness, with statistical differences between basal vs. placebo (p=.001) and basal vs. P144 (p=.004). When comparing placebo vs. P144,

there was a clear trend of smaller total area and reduced thickness although this difference did not reach statistical significance. Fig. 3 and 4.

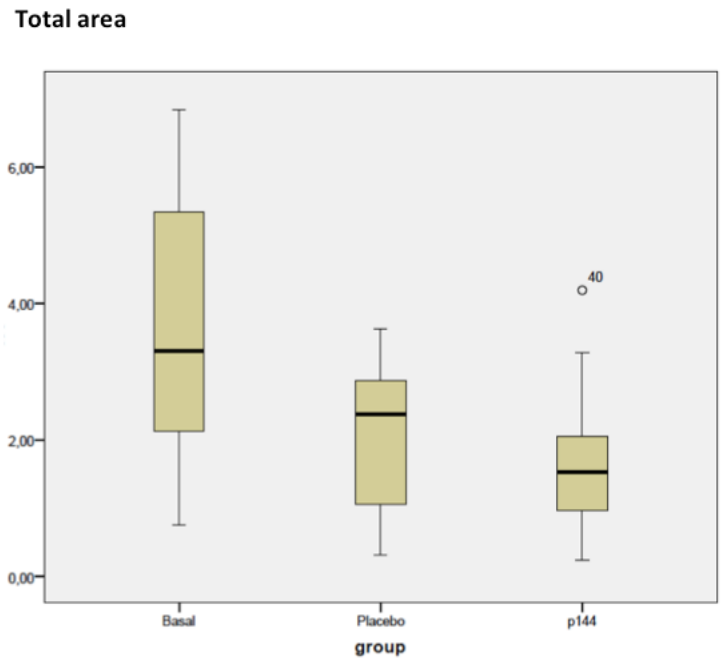

Fig. 3. Box plot with the results of total area in the three groups. Measure in cm^2

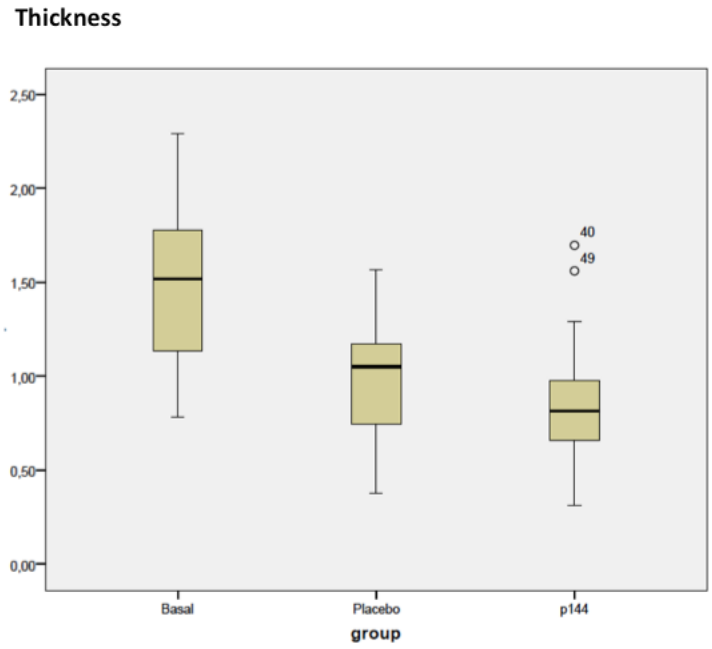

Fig. 4. Box plot with the results of thickness in the three groups. Measure in cm

3. Effect of the treatment in collagen fibers

3.1. Morphometric analysis of collagen fibers area

Table 2 shows the results of collagen fibers area in the comparison between the groups. Statistical significance was found.

Table 2. Comparison between the groups in collagen fibers area

Variable	Group	N	Mean	Std. Deviation	*p* value
Collagen area (cm^2)	Basal	18	2.65	1.01	.012
	Placebo	18	1.74	1.10	
	P144	18	1.49	1.01	

In the subgroups comparison, basal vs. placebo (p=.022) and basal vs. P144 (p=.028) showed significant differences. In the comparison between placebo vs. P144 the collagen fibers area trended to be smaller in the treatment group, although the difference did not reach statistical significance. Fig. 5.

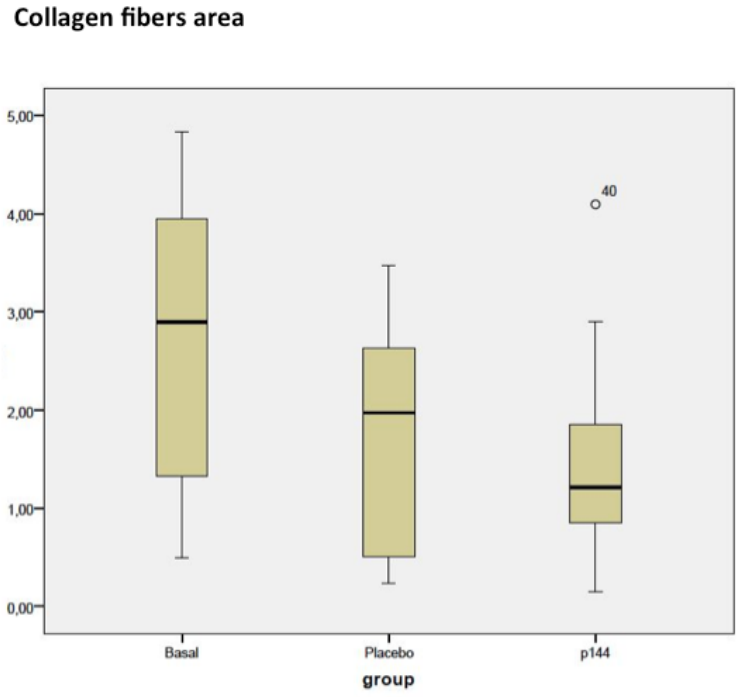

Fig. 5. Box plot with the results of collagen fibers area in the three groups.

3.2. Morfological features of collagen proliferation

Table 3 shows the results of the collagen proliferation in the comparison between the groups. Statistical significance was found. Two blinded pathologists labeled dermal collagen proliferation comparing pairs of same scar implanted in mice after receiving placebo or treatment with basal group.

Table 3. Comparison between the groups of collagen proliferation

Variable	Group	N	Mean	Std. Deviation	*p* value
Collagen proliferation	Basal	18	2.61	0.97	
	Placebo	18	2.33	0.91	.012
	P144	18	2.00	0.84	

In the subgroups analysis, significant difference was only found between basal vs. P144, with p= .026. The differences were not statistically significant in neither comparison between basal vs. placebo nor placebo vs. P144. Fig. 6.

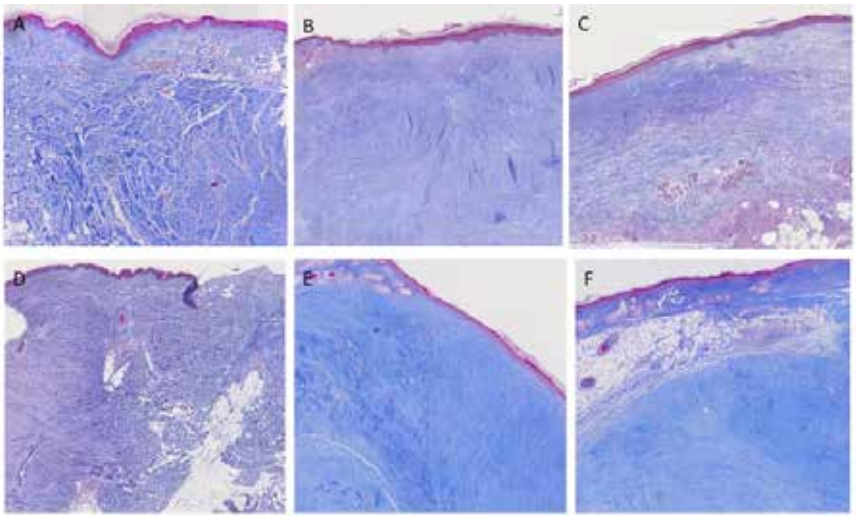

Fig. 6: Histological preparations of the hypertrophic scars staining with Masson's Trichrome. (20x magnification). A. Basal group (no implanted scar). Intense dermal collagen proliferation with thickened and rearranged collagen bundles. Epidermis maintains its normal features and an absence of skin appendages is observed; B. Same scar of A, after excision from the mouse, placebo group. Very intense dermal collagen proliferation with derangement of the collagen fibers. Epidermis resembles human epidermis with absence of skin appendages; C. Treatment group from the same scar. Moderate dermal collagen proliferation with normal human epidermis; D. Basal group. Hypertrophic scar with very intense dermal collagen proliferation with engrossed and disarranged collagen bundles; E. Placebo group. Moderate dermal collagen proliferation with human epidermis preserved; F. Scar from the treatment group, with moderate dermal collagen proliferation and human epidermis features.

3.3. Immunocytochemistry staining of collagen I and collagen III

Table 4 shows data from the immunohistochemistry results. Statistical reduction of Collagen I in both implanted scars compared with the basal group were found. The change of Collagen III in the three groups did not reach statistical significance. In the subgroups analyses, no statistical difference was found comparing P144 group with placebo in any of the variables. However, a trend of lower levels of collagen III was found in the treatment group. Significant difference was observed when comparing basal vs. P144 in the collagen I (p=.003) and basal vs. placebo (p=.002). Fig 7 and 8 show some examples of hypertrophic scars stained with anti-collagen I and anti-collagen III.

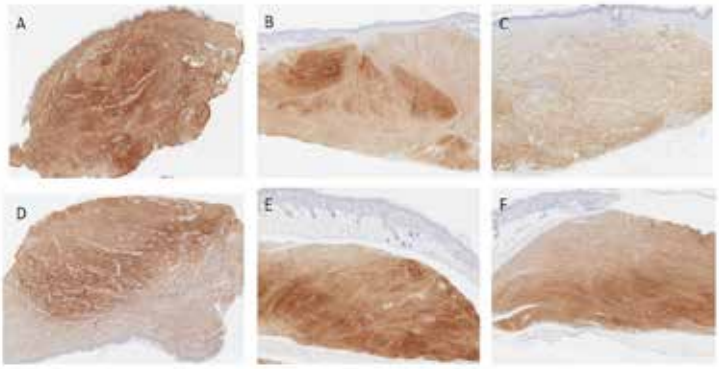

Fig. 7: Images of hypertrophic scars staining with anti-collagen I. A (basal), B (placebo) and C (treatment) belong to the same scar. D (basal), E (placebo) and F (treatment) are from another scar. Fewer stained fibers can be seen in C and F when comparing to the other groups.

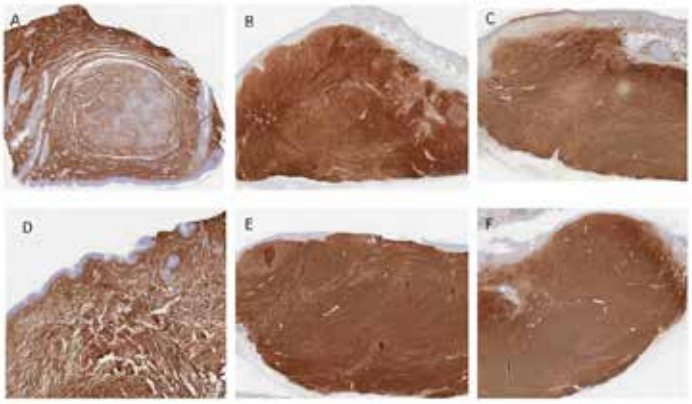

Fig. 8: Images of hypertrophic scars staining with anti-collagen III. A (Basal), B (placebo) and C (treatment) represent the same scar. The differences between these three groups are minimal. The same happens with the second scar, D (basal), E (placebo) and F (treatment).

Table 4. Comparison between the groups of collagen I, collagen III, and ratio Collagen I/ Collagen III

Variable	Group	N	Mean	Std. Deviation	Std. Error Mean	*p* value
Collagen I (Ratio%)	Basal	18	70.16	16.44	3.87	
	Placebo	18	49.72	20.84	4.91	.005
	P144	18	50.5	20.88	4.92	
Collagen III (Ratio %)	Basal	18	67.5	23.88	5.63	
	Placebo	18	61.8	24.08	5.67	.163
	P144	18	58.5	20.06	4.73	
Col I/Col III	Basal	18	1.82	2.75	0.64	
	Placebo	18	1.14	1.32	0.31	.160
	P144	18	1.18	1.34	0.32	

4. Effect of the treatment in elastic fibers

Table 5 shows the results of the comparison between groups in the staining of fibrillin-1. Significant difference was found and it was remarkable the difference between basal group and P144 and placebo. In the subgroups analysis, basal vs. placebo (p=.002) and basal vs. P144 (p=.003) showed significant differences. However, placebo vs. P144 showed a trend of higher amount of fibrillin-1 in P144 group without reaching statistical significance. Figure 9 shows fibrillin-1 expression in the same scar of the three groups.

Table 5. Results of the comparison between the groups of fibrillin-1

Variable	Group	N	Mean	Std. Deviation	Std. Error Mean	*p* value
Fib-1 (Ratio%)	Basal	18	1.88	2.587	0.61	
	Placebo	18	27.33	16.26	3.83	.000
	P144	18	32.77	17.15	4.04	

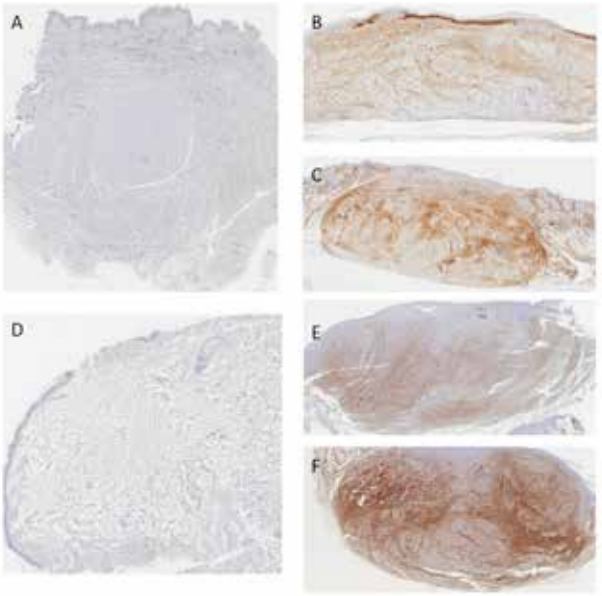

Fig. 9: Images of hypertrophic scars staining with anti-fibrillin-1. A (basal), B (placebo) and C (treatment) belong to the same scar and D (basal), E (placebo) and F (treatment) to a second one. It is noticeable that there is an absence of elastic fibers in A and D (basal group).

DISCUSSION

1. The animal model

The "in vivo" model of human hypertrophic scar implanted in nude mice has proved to maintain the histotypic characteristics, viability and scar morphology along the duration of the present study. This finding is supported by Shetlar et al. study[133] results with implantation of keloids in nude mice. They found that all tissue specimens preserved the morphology and glycosaminoglycan distribution for at least 60 days after implantation. Shed hypertrophic scars demonstrated good vascularization and with morphological and histological features that resemble human hypertrophic scars. Kischer et al.[144] presented similar results after implantation of hypertrophic scars in nude mice. They observed that all hypertrophic scars and keloids retained their typical nodular characteristics.

This method might be one of the closest to the clinical scenarios since a true human hypertrophic scar provided with intact epidermis is shed on mice.

Probably the novelty of the current method is the implantation of each scar in two different mice, preserving a sample of each tissue without implantation as basal group. One of the mice received placebo whereas the second one received treatment. This grouping tries to replicate a case-control study, providing a matched control for each case. This technique allows for studying the effect due mainly to treatment or resulting of implantation since the basal differences were minimized. There is no doubt that some slight differences may still exist seen as each animal has its own peculiar biological response under same conditions. Previous studies used other methods in the attempt to overcome these basal differences. Xie et al.[145] implanted two hypertrophic scars in the same mouse to test a new therapy. The preservation of a third piece (basal group) from each scar used in the current study allows for later comparisons between the implanted and not implanted scars. The behavior of these scars after implantation, morphological and histological changes as well as the progression of healing process are revealed with more clarity when data from preimplantated scar status is available for comparison.

One of the drawbacks of using nude mice is the length of the experiment, since their short life span limits the duration. Because of the T-cell deficiency they are more susceptible to suffer from neoplasms, glomerulosclerosis, cardiac calcinosis during long studies[141]. In addition, other factors may interfere in the study outcomes, such as diet, housing, infectious agents, light cycle and temperature. For these reasons, the majority of studies with nude mice last from of few days[146-148] to several weeks[149]. Economical issues are also involved since nude mice need to be placed in individual cages provided with positive pressure and all manipulation has to be done under laminar airflow cabinets. Based on previous studies results using other animal models, the length of the current experiment was set at two weeks of treatment. Two-week treatment has demonstrated to be sufficiently long to obtain significant results in a study using topical P144 gel in an "in vivo" model with C3H mice[120]. A different study conducted by Zhao et al.[37] tested the efficacy of transdermal patch with an inhibitor of TGF-β1 and significant results in lower levels of TGFβ1 protein were observed after two weeks of treatment. Irradiation with high-dose ultraviolet light[104] in rabbit ear model during 7 to 14 days reduced the scar volume. Again, it seems that two weeks is enough time to determine whether a therapy is effective or not.

The mean shedding period of the current study was 35±5.4 days. Compared with the time reported by previous studies using the same method[150] (around 15 to 20 days), this was slightly longer. In this study, hypertrophic scar grafts were fixed to surrounding tissue with sutures. Although the grafts were healed before, application of lipogel containing either placebo or P144 could not start until the sutures were spontaneously removed. In doing this, the animals were spared to receive a new dose of anesthetics and additional manipulation to remove all stitches in the laminar airflow cabinet. For this reason, in the present study, the time considered as shedding period was determined as the time when all stitches were spontaneously removed from the graft. Obviously, it was a bit longer than previous similar studies using human hypertrophic scars xenografts.

Regarding implantation of fibrotic scars into nude mice, some authors[135] states that since these tissue samples have undergone ischemic process when released from the patients, after shed in the animals their scarring process does not contain the initiating factors that originally led to the development of the hypertrophy. Contrary to this point of view, Kim et al.[151] found that an over-expression of TGF-β receptors type I and II can be detected up to 2 years post injury in the implanted scars. Moreover, according to numerous studies regarding

fibrotic disorders, not only the initiating factors are crucial for the development of hypertrophic scarring but also the existing inability of other molecules to downregulate the activity of TGF-β in fibroblasts increases the magnitude of this cytokine modulation. Based on the aforementioned results, treatment with inhibitors of TGF-β1 may still have effect in chronic pathological scars because its target is still present.

Initially the sample size was set up at 60 animals however during the time following tissue samples implantation some animals died, likely due to infection. For this reason, the effect of the treatment was finally studied in 36 animals. Previous similar reports using nude mice included a range from 18[130] to 48[152] animals to study different therapies behavior. In fact, in the firstly mentioned study they found statistically significant results in spite of the small sample (hypertrophic scars from 9 patients) after 4 weeks of treatment.

In order to further understand in detail the mechanism involved in the pathogenesis of hypertrophic scarring, "in vitro" models using cultured fibroblastic cells have been widely used for this purpose[40]. By means of reproducing controlled environment with ideal conditions, extensive studies can be conducted to determine the role of some cytokines[31,49,153] in fibrotic scarring

process and regulation of extracellular matrix metabolism. These studies provide in-depth knowledge of the scarring process given that each regulator can be separately examined, thus offering accurate understanding of its mechanisms and its contribution to hypertrophic scarring. However, “in vitro” environment may be particularly restrictive in reproducing the complex system that leads to extracellular matrix production, deposition, turnover and degradation. This sort of study design fails to reproduce a dynamic and complex process that constitutes the main characteristic of wound healing.

Therefore, the necessity of an adequate “in vivo” model that resembles that complexity is clear. A more holistic approach has to be created to allow the analysis of a wide range of molecule interactions and to elucidate which mechanisms lead to over-expression of pro-inflammatory signaling. Abnormal wound healing results from imbalance between pro-inflammatory and anti-inflammatory cytokines and other regulators. Neither the study of the scar development process nor the recapturing of the pathogenesis of hypertrophic scarring can be achieved through the currently known “in vivo” models.

There is still a long way to fully understand the mechanisms behind the accumulation of excessive extracellular matrix components that could potentially

aid in developing hypertrophic scarring. An ideal animal model is key in addressing the former issue and may be the principal way to overcome some weak points in the knowledge of the broad field of fibrotic disorders.

2. Macroscopic changes of the hypertrophic scars after implantation

Total area and thickness of the scar were the outcomes chosen to assess the macroscopic changes in the current study. Globally, a significant decrease was found in both total area and thickness of the scar when comparing either placebo group or P144 group with the basal group. These findings suggest the possibility of shrinkage or contracture that the scars undergo after the implantation and shedding period. The latter may be a common behavior encountered when performing skin grafting in patients[154], more remarkable for split thickness skin grafts than for full thickness. In addition, size reduction of the implanted scars in time was supported by a previous study[155]. Its authors described a volume reduction in all implanted hypertrophic scars and keloids in nude mice. In the current study this reduction in total volume and thickness was quantitatively

assessed because data from preimplanted status of each scar was available for all used scars.

A clear trend of decreased total area was found when comparing treatment vs. placebo group. Although this difference did not reach statistical significance, a difference of 3.4 mm^2 may have clinical relevance in scars of $20mm^2$ (17.3% of total area reduction). The difference in thickness was smaller, of 1.1 mm (approximately 10% of the placebo group mean thickness) although also favors the treatment group. In addition, because of shrinkage and contracture phenomena aforementioned that affect all implanted scars, major differences are required to achieve statistical significance.

In the majority of animal studies macroscopic changes are rarely considered one of the main outcomes. Only few of them assess the thickness and volume of the hypertrophic scar before and after the treatment. In the study of Yuan et a.[111] they found a decreased in collagen I production associated with flattened dermis after ASODN (antisense oligodeoxynucleotide) treatment. Lowering in scar elevation was measured in rabbit ear models[98-100] after subcutaneous injection of different agents. In other study using the same model, scar volume reduction after high-dose ultraviolet light[104] was observed. Apart from

the first of the abovementioned studies, no other has used human scars to test the treatment. Since these emergent therapies would be susceptible of having clinical application, the choice of animal model should be one that resembles more closely the clinical scenario. In spite of the well-known limitations of the "in vivo" model of human hypertrophic scars implanted in nude mice, this model exhibits many similarities and few disparities with the hypertrophic scars in patients[156] and allows for the assessment of macroscopic features changes.

Unlike animal studies, among the studies conducted in patients to test different therapies, the main outcome of the majority of them is related to either reduction of scar elevation, improvement of the appearance and color, decrease in thickness, increase in elasticity or reduction in hardness. There is no doubt that effective treatment should result in clinical improvement. Animal studies are the previous step necessary for testing safety and doses of new therapies, and they should resemble human hypertrophic scars acutely to allow extrapolation of results. In spite of the volume reduction experienced in implanted scars, direct implantation of human hypertrophic scars in nude mice may be one of the most suitable models for measurement of macroscopic changes due to treatment.

3. Scar maturation after implantation and treatment

Histological and biochemical characteristics of human hypertrophic scars implanted in nude mice have been reported in terms of levels of glycosaminoglycan[133] and histological appearance[150]. The data available was extracted from a few studies where the authors studied the changes observed in the implanted scars compared with their pre-implantation status. In order to contribute to this knowledge, this study is provided with a basal group that enables to determine what kind of changes the implanted scars undergo and whether changes are due to implantation itself or treatment. As observed in the current study, histological changes are consistent with possible reversion toward normal healing process due to topical application of P144.

The grafts take rate was high in this study, 50 out of 60 scars (83.3%) shed completely. In five placebo cases, granuloma on foreign bodies was found secondary to surgical sutures. Mild cell infiltration and proliferation was found and it was considered a normal sign during shedding process.

Histological images of the implanted scars show features of human hypertrophic scar provided with an intact human epidermis attached to the

thickened dermis. Complete absence of skin appendages was found in all cases and this also happened in the basal group. Boundaries between papillary and reticular dermis were diffused. In the dermis layer of the scars, disarrangement of collagen bundles was noted, irregularly distributed in the reticular dermis and forming nodular, circular or whorled configurations. The former features could be clearly distinguished from the surrounding mouse skin, in the form of one-layer epidermal cells with numerous hair follicles at the dermis. At the edges between human and mouse skin no signs of acute rejection were found. In some cases human epidermis was partially lost during the healing process so mouse skin healed with the scar borders. In transversal sections the scar could be seen partially or totally embedded beneath mouse skin.

Immunohistochemistry results showed significant decrease in collagen I expression and increase in fibrillin-1 in the groups' comparison. Statistical differences were noted between basal vs. placebo group and basal vs. treatment group. Consequently, these findings happened in all implanted scars. Excessive amount of collagen type I accumulation contributes to the formation of hypertrophic scar[131] and at the end of the wound healing process, this replaces collagen III fibers, which are produced more prominently in the early phases[157]. A

decrease in collagen I may be due to the scar maturation process itself. It is known that collagenolysis by activated MMP and cell apoptosis are promoted at the remodeling and resolution phases[21] and this results in a mature scar. Changes achieved by the treatment are not significant in comparison with placebo group likely because reduction of the collagen amount is not an expected consequence of inhibition of TGF-β1 modulation. Among the pro-fibrotic processes regulated by this cytokine are the promotion of collagen synthesis by enhancing activation and proliferation of fibroblast[31], spurring fibroblasts differentiation to myofibroblasts[18] and favoring extracellular matrix proteins deposition[112]. Collagenolysis may not be facilitated by inhibition of any of these mechanisms. Taking into account that all scars used for the present study had at least 6 months of evolution since the injury, it was supposed that they would be in the remodeling phase and the pro-inflammatory signaling would have been weakened already. Collagen production at this stage could neither increase nor be increased after the implantation into nude mice. In fact, in a study of burn wounds[158] the authors demonstrated that collagen synthesis reaches its peak 6 months after injury and starts to present a normal turnover rate 2 to 3 years after healing. Therefore the decrease level of collagen I in both placebo and treatment groups seen in the current study is likely due to normal collagen breakdown during scar maturation process.

The amount of collagen III was similar in the three groups, either in the samples before implantation or after implantation, most likely due to an absence of its production and lack of its replacement by collagen I. As it has been mentioned before, the scars included in the present report were excised at least 6 months after injury and therefore the expression of collagen III by fibroblasts was likely minimalized[159]. It is presumed that inhibition of TGF-β1 activity decreases extracellular matrix components deposition, especially the synthesis of collagen[33]. Collagen degradation was quiescent, leading to lack of change in its amount along the treatment period since inhibition of TGF-β1 did not enhance collagenolysis. Previous studies[160-162] in burn wounds support these findings, given that they found that content in collagen did not decrease at 18-24 months compared with the amount of collagen found in the same scars at 1, 3, 6 and 12 months. A possible reason for that may be that, although synthesis and catabolism might be in balance after 12-18 months from injury, an excess of collagen in the scars persists due to its over-production in the early phases of wound healing. An active collagenolysis should be induced in order to achieve a decrease in collagen fibers deposition, as even after elimination of its synthesis promoters, the amount of collagen remains higher than in normal scars.

Imbalance between synthesis and degradation of collagen is a common sign of fibrotic conditions such as hypertrophic scars and keloid[163]. The assessment of collagen I/III ratio may be helpful to determine whether excessive skin fibrosis is present. Many factors have demonstrated their influence on the amount of collagen deposition, not only expression of collagen genes[26] but also the patients' age[164]. According to the former study, collagen I/III ratio in hypertrophic scars was shown to be lower in patients under 20 years of age compared with 20-50 years old patients (average of 3.76 to 6.48). Friedman et al.[26] found that keloids presented a ratio of collagen I/III of 17:1 whereas normal scars had 6:1. Although hypertrophic scars and keloids share many common morphological features, differences in the collagen metabolism were found according to Friedman's study. The authors stated that in keloids, the expression of α1 procollagen gen was correlated with an increase of α1 procollagen mRNA levels whereas in hypertrophic scars, posttranscriptional mechanisms were able to down-regulate the elevated levels of mRNA and consequently, decrease the synthesis of collagen. Since hypertrophic scars are provided with different collagen content than keloids, the collagen I/III ratio for keloids cannot be equally applied to hypertrophic scars. The collagen I/III ratio found in the current study has an average of 1.82 for basal group, 1.14 for placebo group and 1.18 for

treatment group, with no statistical differences. An increase in this ratio might be expected with later replacement of collagen III by collagen I. Since no changes were reported in collagen III fibers, it was supposed that after time and maturation of the scar, this replacement would take place and the collagen I/III ratio would increase to become similar to normal scars.

Increased staining of fibrillin-1 found in both placebo and treatment group may also be considered another hint of progressive scar maturation[142]. According to Costa et al study[27], in hypertrophic scars, the clinical improvements occurred as a result of rearrangement of elastic system components. The disturbance of elastic fibers system results in impairment of extensible and dynamic connective tissue properties with lack of long-range elasticity[29]. Clinically, this explains why hypertrophic scars are typically presented as hard, stiff and immobile lesions. In all implanted scars, decrease in collagen I and increase in fibrillin-1 shows a maturation of the scar after being implanted in the mouse compared with the basal condition. The difference between placebo and treatment in fibrillin-1 staining did not reach statistical significance although a trend towards higher levels of the former in treatment group was found. Results showed a difference of 16.5% between placebo and treatment group. These results may support those obtained in

total area of scars. The reduction of 17% in total area found in treatment group compared with placebo group might be partially induced by more complete re-establishment of elastic fibers system in the P144 treated group. With the inhibition of TGF-β1, it seems that wound healing process returns to its normal course, allowing the rearrangement of elastic fibers and recovering of normal scars elastic properties. As it was mentioned above, this process may occur in a normal healing process. However, it may be likely enhanced with the inhibition of TGF-β1. According to previous studies[143], over three months after injury, a progressive increase in elastic fibers is present. Curiously, in all of the tissue samples from the basal group, elastic fibers content was minimum. In fact, comparing the same scar from basal (pre-implanted) to implanted scars, great changes are observed. An increase of 94% of fibrillin-1 expression was observed comparing basal with treatment group. This phenomenon supports the active role of P144 in the reversion towards normalization of the implanted scars healing process.

4. Topical application of treatment

Since hypertrophic scars constitute a chronic condition, the way of treatment administration should be carefully chosen because patient adherence to therapy is paramount to achieve clinical improvements. A clear trend so as to decrease harmful procedures is noticed lately in the strategies against hypertrophic scars. Topical and other non-invasive ways of administration are preferred to those more invasive such as intralesional injections or surgical resections.

Testing new therapies in animal models using topical application may lead to erroneous results because of uncertain absorption rate. In spite of this drawback, this way of administration has already been used to examine the efficacy of many different treatments. Topical zinc oxide[92] was tested in rabbit ear model by daily application for 3 weeks and clinical scar hypertrophy decreased after completion of treatment. Basic FGF[93] was also tested in rabbit ear model because of its effects on collagen synthesis inhibition and enhancement of collagenolysis. After 3-times per day treatment for one, two and three weeks, improvement was found in the hypertrophy. The application of cellulose gel in addition with recombinant stratifin or ASA[95] also led to reduction of scars in

rabbit ear model. Similar results were obtained with topical application of indoleamine 2,3 dioxygenase[96] in rabbit ear. Topical P144 lipogel has already shown its efficacy in decreasing soluble collagen content in a scleroderma model in mice[120]. To sum up, topical therapies recently tested in animal models are showing promising results. There is no doubt that the results may be susceptible of bias since the absorption rate is different from one animal to another. Another backbone of topical administration is the necessity of an animal model provided with intact human epidermis above the scars to avoid structural differences that may have impact on the absorption making it difficult to extrapolate the results. Histological morphology of human and other animals differs greatly. Nude mice are provided with monolayer epidermal cells with scarce stratum corneum at the top of the epidermal layer whereas human epidermis is a multilayer of keratinocytes with thick stratum corneum. Human hypertrophic scars implanted in nude mice may be one of the most suitable "in vivo" models to test topical agents because it features intact human epidermis resulting in small potential difference in absorption rate compared with patients.

The preference for topical application of TGF-β1 inhibitors over systematic administration is progressively increasing after the forementioned

studies[37,165] where they demonstrated that the topical way was superior to others. In fact, the authors found that inhibitors of TGF-β1 had short half-life, complex mechanism and low content after systemic medication. For this reason, topical application of these modulators of TGF-β1 activity may increase and maintain their half-life with optimal level for a longer period of time.

5. New horizons in treatment of hypertrophic scars

5.1. Superficial dermal fibroblasts

Depth and location of wounds are believed to be the main factors in hypertrophic scar development[166]. Since fibroblasts play a crucial role in wound healing, these cells might be provided with different properties depending on their location. Studies have shown the existence of at least three subpopulations of fibroblasts with unique phenotypes and functions[167]. Fibroblasts are labeled as superficial dermal fibroblasts, reticular or deep dermal fibroblasts and fibroblasts associated with hair follicles[46]. Superficial and deep dermal fibroblasts exhibit different physical and biochemical characteristics[168] in cultures. Cells from deeper layers proliferate at a slow rate and produce more TGF-β1[46], collagen type I and versican, but less decorin. In addition, collagenase expression is decreased in these

cells[39]. Several studies state that deep dermal fibroblasts behavior might be more similar to fibroblasts found in hypertrophic scars[46,169] than those found in superficial layers.

In presence of injury, an increased migration of fibroblasts to the wound bed may favor an optimal wound healing given that these cells promote faster wound closure and reepithelialization[170]. Unfortunately, in deeper wounds, migration of deep dermal fibroblasts toward upper dermal layer may not be beneficial for the healing process since the latter might promote excessive extracellular matrix production and accumulation leading to fibrotic scarring.

In the light of the aforementioned studies, the possibility of isolation and transplantation of superficial dermal fibroblasts to wound bed may prevent hypertrophic scar development after full thickness injuries. Honardoust et al.[113] showed some conclusions supporting this hypothesis since they found that superficial wounds heal with little or no scar formation whereas deeper wounds were more prone because of a larger number of activated reticular fibroblasts. The challenge will reside in the way to isolate autologous superficial fibroblasts and how to create a proper environment for the survival of these cells in the wound bed. This therapy would probably be promising since transplantation of

autologous superficial fibroblasts cells to the wound bed may decrease the incidence of hypertrophic scars formation. Fibroblasts sensibility to TGF-β modulation has been considered the main body of work in the development of hypertrophic scars. It is believed that the influence of TGF- β on superficial dermal fibroblasts, contrary to fibroblasts from deep dermis, is weaker[20]. Predisposing factors that facilitate this sensitivity are not well established yet, and there is, therefore a lack of evidence to determine the risk of hypertrophic scarring development. Deeper knowledge in this field may be worthy because, theoretically, scars appearance would improve in the presence of superficial dermis fibroblasts.

5.2. Decreasing wound tension

In prevention of hypertrophic scars formation, decreasing wound tension represents one of the most important modifiable factors during proliferative and remodeling phases[171]. The placement of surgical incision should be thoughtfully chosen to prevent high- tension areas after closure.

Barbed suture has been used since 2005, when it received US Food and Drug administration approval for soft tissue[172]. It can be placed in superficial and deep fascia, cartilage, tendon, joint capsule, fibrous periprosthetic capsules and

skin. This sort of suture consists of a barbed absorbable thread, armed with a surgical needle at one end and a loop end effector at the other. It is provided with barbs and loop end effector that allow for tissue approximation without the need to tie surgical knots. Barbed sutures composition is prepared from asynthetic polyester composed of glycolide, dioxanone and trymethylene carbonate[173]. Advantages reported from previous studies state that it reduces operative time and provides better wound closure than conventional sutures[174], demonstrated for lipoabdominoplasty[173]. The successful use of barbed sutures in the improvement of scar cosmesis and its suitability for closure of high-tension wounds in a variety of surgical settings are the reasons why they are becoming more popular and essential every day.

The use of barbed sutures may prevent excessive tension in long and tensional wounds providing adequate wound closure with one-layer suture fashion. Further studies are warranted to find histological changes comparing the former with conventional sutures regarding scar development under both conditions. Recovery of balanced mechanical forces after wound closure might be beneficial in the function and cosmesis of the final scar.

5.3. Deeper understanding of fetal wound healing

Several studies have been conducted in the last decades in the attempt to understand fetal scarless healing. Given its unique peculiarities, fetal healing process is considered an attractive and emergent line of research since deeper knowledge in this field may help to avoid hypertrophic scar development in adults. However, fetal skin is surrounded by different environmental conditions than adult skin, such as no air-exposed skin.

Fetal wound model has demonstrated to have faster reepithelialization rate[175] than in adult skin models and this might be partially responsible for a better scar outcome. The relationship between healing time and hypertrophic scar formation is well understood in burned patients[166]. It has been hypothesized that inflammatory phase in fetal skin is auto limited and production of collagen is down regulated after complete reepithelialization[176]. This situation leads to decrease in collagen production and deposition by fibroblasts and theoretically to prevention of abnormally fibrotic healing. Previous studies support that hypertrophic scars consist mainly in an excessive amount of type I collagen accumulation[177], therefore selective inhibition of collagen production might prevent their formation. In addition, TGF-β1 profibrotic signaling in the early

healing phases[22] is crucial for hypertrophic scarring and its early inhibition could decrease the later cascade of cytokines and cell activation toward pro-fibrotic response.

In fetal healing model, a higher number of fibroblasts was found in the early healing process than in adult skin. It allows the rapid production of extracellular components, such as fibronectin and tenascin, which contribute to a fast reepithelialization and cell migration[178]. After 7 days, the number of proliferating cells decreases significantly and no myofibroblasts are detected[179]. According to previous studies in patients, the presence of myofibroblasts in wound bed was considered partially responsible for hypertrophic scar formation[180] so their absence should benefit a normal healing process.

In the light of studies in fetal wound healing, a faster reepithelialization may lead to an auto limited inflammatory phase preventing hypertrophic scarring formation. Ideally, emergent therapies should be able to reduce the time and magnitude of the inflammatory response after wounding. New therapies may attenuate myofibroblast differentiation but are unlikely to achieve total suppression of them.

5.4 Prevention better than treatment

The majority of the studies regarding pathological wound healing stated that detailed knowledge of the mechanisms behind this process remained not well established. Efforts should continue seeking for adequate experimental models that may resemble human hypertrophic scar including its pathogenesis and development processes. However, optimistically, the broad knowledge acquired in wound healing process provided with finely regulated components and signaling transduction cascades that modulate determined genes expression, is a very valuable knowledge.

Most of the studies agree that there is an association between prolonged inflammation and hypertrophic scar development. Cytokines are considered the main regulators in the early phases of healing process and TGF-β1 is one of the most representative[116] as it has already been mentioned. Not only because of its role in modulating numerous pathways promoting synthesis of collagen and fibroblasts proliferation but also due to its ability to induce its own production, resulting in prolonged pro-fibrotic response. Overproduction of extracellular matrix components associated with diminished production of collagenase lead to excess of collagen deposition and development of hypertrophic scars.

On the basis of previous reports[177,181], imbalance in collagen metabolism is mainly due to over production. Moreover, other studies[182] support a decreased expression of mRNA collagenase in hypertrophic scars fibroblasts. For these reasons, inhibition of collagen synthesis might achieve better outcomes than promotion of collagen degradation. Other studies regarding long-term burn wounds found persistent excess of collagen remained although modulators have disappeared[161]. Therefore, theoretically further benefits would be achieved promoting inhibition of collagen production that takes place mainly at the early phases of the healing process than enhancing collagen degradation at later phases. Inhibition of excessive inflammatory response, with down-regulation of those pro-inflammatory cytokines in the inflammatory phase might decrease collagen production and improve final scar outcome. Consequently, the proper time for application of preventive agents should be within the first weeks after wounding. Further studies are warranted to infer predisposing factors in patients so as to determine which one would benefit most from preventive action.

CONCLUSIONS

1. A clear trend of macroscopic reduction was observed on the implanted scars, more noticeable in the treatment group compared with placebo group although the real magnitude of its effect may be underestimated.

2. The most remarkable effect of P144 topical treatment on hypertrophic scars was the induction of faster scar maturation evidenced by a decrease in collagen content and rearrangement of the elastic fibers system.

3. According to the obtained data in this study, P144 could be used for human hypertrophic scars treatment.

CONCLUSIONES

1. En el grupo del tratamiento se observó una clara reducción de las variables macroscópicas de las cicatrices en comparación con el grupo placebo. Sin embargo, debido a que la propia implantación produce este efecto, la magnitud real debido al tratamiento pudo estar subestimada.
2. El efecto más llamativo secundario al tratamiento tópico con P144 fue la reversión a un proceso de cicatrización normal, evidenciado por una disminución del contenido en colágeno y en una reordenación de las fibras elásticas presentes en la dermis.

3. De acuerdo con los resultados obtenidos en el presente estudio, P144 podría ser útil en el tratamiento de las cicatrices hipertróficas en pacientes.

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