c-myb proto-oncogene is up-regulated in hypertrophic scars and correlates with increased collagen I

Kopecki Z, Adams D & Cowin AJ

Abstract

Hypertrophic scar formation is a serious medical problem involving an excess fibro-proliferative response; it is particularly observed in burns patients and patients with a variety of diseases including keloids and scleroderma. Type I collagen is the major form of collagen produced in response to wounding and is involved in cutaneous fibrosis and scar formation. A recently discovered function of the well known c-myb proto-oncogene, previously reported to be involved in regulating cell growth and development, is its ability to regulate type I collagen protein production. Previously we have shown that c-myb plays an important role in wound repair, by regulating collagen synthesis and cellular proliferation and migration. Here we examine the involvement of c-myb in different human wound and scar tissues. Human tissue collected from hypertrophic scars, keloid scars, acute wounds and chronic venous leg ulcers were immunostained for c-myb and collagen I.

Our results reveal a significant increase in c-myb protein levels in hypertrophic scars but not acute wounds, keloids or chronic ulcers. Interestingly, this increase in c-myb expression corresponded to the increased expression of collagen I in hypertrophic scars. These findings suggest that c-myb may be an important modulator of collagen synthesis in hypertrophic scar formation and may be important in wound fibrosis and scarring. Manipulation of c-myb levels may be of value in promoting improved wound healing and reducing scar formation.

Introduction

Fibrosis, or scar formation, is the final endpoint of the cutaneous wound healing process seen in both adult humans and higher vertebrate animals. Tissue repair by fibro-proliferative response leads to excessive fibrosis and results in undesirable scar formation; this can range from a few fine lines to hypertrophic scars and contractures most often seen post-burn injury [1].

Numerous studies have examined the changes that take place in the remodelling phase of wound repair which lead to replacement of granulation tissue by fibrotic tissue. Main events that occur involve changes in the extracellular matrix which include increases in the deposition of type I collagen, wound organisation and contraction and decreased numbers of vessels and myofibroblasts by processes including apoptosis [2, 3]. The deposition of collagen type I is important for scar formation, and proteins that may regulate this process have potentially important functions in modulating cutaneous fibrosis.

A recently discovered function for the c-myb proto-oncogene, previously known to be involved in the regulation of cell growth and development, is its ability to regulate type I collagen protein production [4]. Indeed, we have previously shown that c-myb deficiency impairs wound healing and results in decreased collagen I synthesis, therefore suggesting that c-myb may have an important function in scar formation and fibrosis [5].

The myb family of genes was discovered through characterisation of their retrovirally transduced cellular counterparts and today is known to consist of c-myb, A-myb and B-myb which all encode for different proteins [6]. Initially these genes, which are all closely related, were thought to be expressed only in haematopoietic cell lines, where c-myb expression is high in immature haematopoietic cells and declines during cell differentiation. However, these genes have many functions and are vital in regulation of growth, development and apoptosis [7-12].

Kopecki Z
Women's & Children's Health Research Institute
North Adelaide, SA

Adams D
Women's & Children's Health Research Institute
North Adelaide, SA

Cowin AJ
Women's & Children's Health Research Institute
72 King William Road, North Adelaide, SA
Tel: (08) 8161 7077
Fax: (08) 8239 0267
Email: allison.cowin@adelaide.edu.au

Collagen is an important matrix protein in the skin and its production by fibroblasts during wound repair directly influences the formation of scar tissue. At present, the mechanisms which regulate the expression of collagen genes in fibroblasts leading to excessive or low levels of collagen type I deposition are not known; however, c-myb homozygous mouse embryonic fibroblasts (c-myb -/- MEF) show a distinct absence of type I collagen α2 chain gene expression but maintain type III collagen and fibronectin expression, indicating that the absence of c-myb has a specific effect on Type 1 alpha-2 collagen gene (COL1A2) [13]. Moreover, excessive gene expression of c-myb has been observed in scleroderma fibroblasts, suggesting that c-myb protein may be involved in the regulation of the human COL1A2 promoter and hence may play a crucial role in wound healing and scar formation [4, 13].

The COL1A2 gene promoter contains four myb-binding sites (MBS); transactivation assays conducted on both wild and mutant promoter-reporter constructs demonstrate that c-myb can transactivate the COL1A2 gene promoter by binding specifically to all four MBS [13]. MBS-4 is the most important for c-myb transactivation of the COL1A2 promoter as mutation of MBS-4 alone strongly decreases up-regulation of the c-myb protein [13]. Transforming growth factor-beta (TGF-ß), an important collagen stimulating factor, induces type I collagen α2 chain expression in c-myb -/- MEF, hence suggesting that TGF-ß signalling pathway is maintained and that the absence of the type I α2 collagen chain promoter gene expression in MEF is due to the absence of c-myb protein [13].

Based on the important evidence portrayed in current research of the new role of c-myb in controlling the type I collagen expression, we have investigated different wound and scar tissues for the presence of c-myb proto-oncogene.

Materials and methods

Antibodies

c-myb/v-myb sheep anti-human primary antibody was purchased from Chemicon, USA. Anti-collagen I primary antibody was purchased from Rockland, USA. Biotinylated anti-rabbit IgG secondary antibody and biotinylated anti-sheep IgG secondary antibody were purchased from Sigma, CA, USA. Cy3-Streptavidin conjugate used in immunohistochemistry experiments was also obtained from Sigma Chemical Company.

Human wounds

Four hypertrophic, four keloid scars and four acute wounds were excised from patients undergoing reconstructive plastic surgery. Additionally, skin samples were collected from four patients with chronic venous leg ulcers on their first presentation at the wound healing clinic of the Queen Elizabeth Hospital, Adelaide, South Australia. The diagnosis of venous ulcer was based on an ankle brachial index of 0.7, a toe pressure of 40mmHg on the limb of the target ulcer, and absence of diabetes. Biopsies (103mm) were taken under local anaesthetic from the margins of the chronic venous ulcers and included wound margin epithelium and surrounding dermal and epidermal tissue.

The study was approved by Human Ethics Research Committee of the Queen Elizabeth Hospital, and was conducted according to the Declaration of Helsinki principles with informed consent obtained from all patients.

Histology, immunohistochemistry and image analysis

All skin biopsies were fixed in formalin, dehydrated through a series of ethanol washes and embedded in paraffin wax for immunohistochemical analysis. Indirect immunofluorescence staining was used for the detection of c-myb and collagen I proteins with a biotin-streptavidin amplification step for increased sensitivity.

Serial 4µm sections of paraffin wax embedded biopsies were cut onto Snowcoat X-Tra™ slides [Surgipath Medical Industries Inc, IL]. Sections were deparaffinised in xylene and rehydrated through a series of decreasing ethanol dilutions. Antigen retrieval was used to rescue antigens masked by the fixation process. This was achieved by placing sections in Target Retrieval Solution [DAKO Cytomation, Botany, NSW], boiling for 2 minutes, initially in a microwave then heating on medium power for 2x5 minutes, before being rapidly cooled to 50°C on ice. Sections were washed in PBS before incubation with 0.025% trypsin at 37°C for 3 minutes. After further PBS washes, the sections were blocked in 3% normal horse serum for 30 minutes before incubation with anti-c-myb (1µg/ml), anti-collagen I (5µg/ml) antibodies for 1 hour at room temperature. Following several PBS washes, sections were incubated with biotinylated sheep or rabbit immunoglobulins (7.5µg/ml) for 1 hour at room temperature.

Labelled proteins were detected using CY3-conjugated streptavidin [1:400; Sigma-Aldrich, Castle Hill, NSW] by incubation for 45 minutes at room temperature. Sections were mounted in DAKO Fluorescent Mounting Medium [DAKO Cytomation, Botany, NSW] and visualised by fluorescence microscopy. Images were captured using analySIS® software [Soft Imaging System GmbH, Munster, Germany] and the fluorescence intensity determined. Negative controls included replacing primary antibodies with normal rabbit IgG, normal mouse or normal sheep IgG. Non-specific binding was determined by omitting primary or secondary antibodies. All control sections had negligible immunofluorescence.

Statistical analysis

The data were analysed using one-way ANOVA followed by a Tukey all-pairwise post-hoc t-test. A p value of less than 0.05 was considered significant.

Results

c-myb is up-regulated in hypertrophic scars

Human tissue excised from a range of different wound and scar types, including hypertrophic and keloid scars and acute and chronic wounds, was analysed by immunohistochemistry for the specific expression of the c-myb proto-oncogene (Figure 1).

c-myb expression was statistically analysed based on fluorescence optical density measurements. c-myb immunofluorescence staining was weak and predominantly nuclear in the dermal fibroblasts within the granulation tissue in acute wounds, non-healing chronic ulcer wounds and keloid scar tissues. Hypertrophic scar tissue c-myb immunofluorescence staining was observed predominantly in both epidermal keratinocytes and dermal fibroblasts where staining appeared to be both cytoplasmic and nuclear (Figure 1A-D). Importantly, c-myb immunofluorescence staining was significantly elevated in hypertrophic scars, with twice as much staining observed in these scar tissues compared to acute healing wounds (Figure 1E). Chronic, non-healing venous leg ulcers showed reduced expression of c-myb protein compared to the acute healing wounds, and c-myb staining in keloid scars was significantly lower than that observed in hypertrophic scars (Figure 1A-E). All control sections had negligible immunofluorescence; a representative negative control section image is illustrated in bottom left quadrant of the acute wound image (Figure 1A).

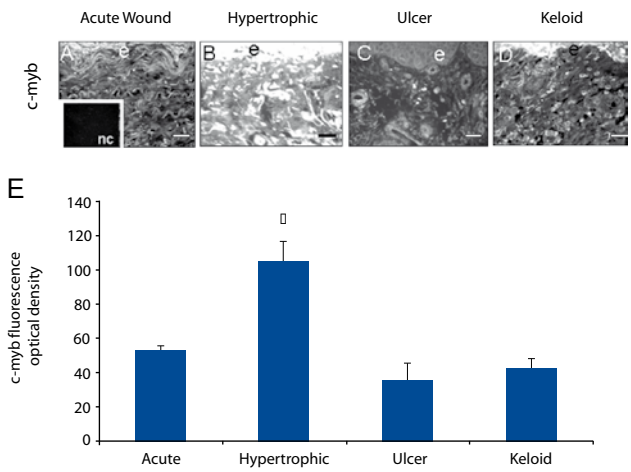

Figure 1. c-myb is up-regulated in hypertrophic scars.

A-D: Representative images of immunohistochemical staining of c-myb in acute, hypertrophic, ulcer and keloid tissues. Scar bar = 100µm.

In all images, e denotes position of the epidermis.

E: Graphical analysis of the c-myb expression based on fluorescence optical density of human acute, hypertrophic, ulcer and keloid tissues.

The representative negative control (nc) section image is shown in the bottom left quadrant of the acute wound image.

*Results represent means and standard error of mean. N=4 per group. *P<0.05.*

Increased c-myb expression correlates with increased collagen I expression

Human acute wounds, chronic non-healing ulcer wounds, hypertrophic and keloid scar tissue were assessed for collagen I expression using isoform specific immunohistochemistry. Graphical analysis of the collagen I fluorescence optical density illustrated a strong positive staining of dermal collagen I in all four wound and scar tissue groups, hence clearly labelling the dermal tissue structure (Figure 2A-E). However, significantly increased expression of collagen I was observed in the hypertrophic scar tissue when compared to acute wounds, non-healing chronic ulcer wounds and the keloid scar tissue (Figure 2A-E). All control sections had negligible immunofluorescence; a representative negative control section image is illustrated in bottom left quadrant of the acute wound image (Figure 2A).

Discussion

c-myb is an important regulator of type I collagen and plays an important role in controlling the proliferation of fibroblasts. Our previous results have shown that c-myb plays an important role in collagen synthesis during wound repair where c-myb deficient wounds displayed significantly weaker wound tensile strength than wild-type counterparts, suggesting that c-myb mediated collagen synthesis has a direct effect on wound repair [5]. Our results now reveal that c-myb is increased in hypertrophic wounds which correlates with excessive collagen production in these tissues. Hypertrophic scars following burn or wound injury are a common form of excessive cutaneous fibrosis characterised by the imbalance between collagen synthesis and degradation during wound repair. Continual collagen production and scar deposition in re-epithelialised wounds results in hypertrophic wounds that produce functional impairments due to rigidity as well as pain and cosmetic abnormality.

In current research not many genes have been identified which are regulated by the c-myb protein; however, c-myb regulation of collagen I gene expression is potentially of great significance. Proteins like c-myb that have the

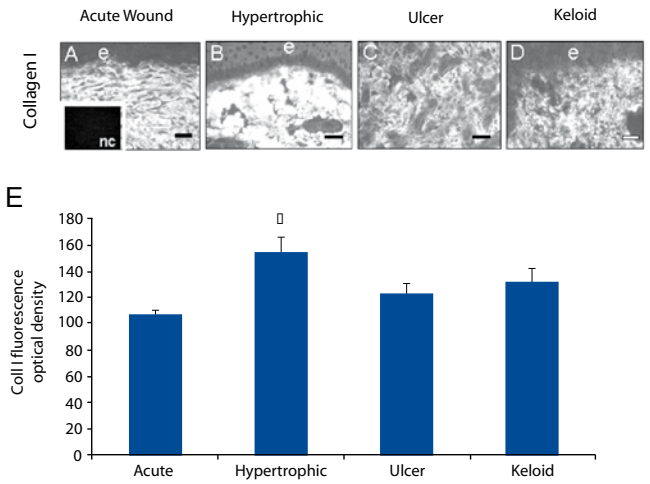

Figure 2. Increased c-myb levels in hypertrophic wounds correlate with increased collagen I expression.

A-D: Representative images of isoform specific immunohistochemical staining of collagen I in acute, hypertrophic, ulcer and keloid tissues. Scar bar = 100µm. In all images, e denotes position of the epidermis

E: Graphical analysis of the isoform specific collagen I expression based on fluorescence optical density of human acute, hypertrophic, ulcer and keloid tissues.

The representative negative control (nc) section image is shown in the bottom left quadrant of the acute wound image.

*Results represent means and standard error of mean. N=4 per group. *P<0.05.*

ability to regulate the collagen expression and deposition during wound repair have potentially important functions in regulating tissue remodelling and scar formation. MBS stability, functional c-myb protein and specific interactions between the DNA-binding region of c-myb and MBS are required for the c-myb transactivation of human COL1A2 promoter [4, 13] which lead to increased collagen production and fibrosis (Figure 3).

c-myb antisense RNA has the ability to inhibit hepatic stellate cell proliferation, α1 collagen and TGF-ß mRNA expression, hence illustrating a key role of c-myb protein in the activation and proliferation of hepatic stellate cells in rats and suggesting possible implications for the treatment of liver fibrosis as well as a potential role of c-myb in regulating and/or interacting with other important mediators of wound healing such as TGF-ß [14].

These observations of a role for c-myb in controlling cell proliferation and an involvement in fibrosis were further documented using scleroderma fibroblasts where high c-myb expression was observed, suggesting that uncontrolled elevated deposition of collagen type I could be linked to c-myb levels and that decreased c-myb expression could perhaps result in decreased collagen type I deposition. Treatment of human fibroblasts with c-myb antisense reduced the expression of α1 collagen and to some extent the α2 collagen expression [4]. Therefore treatment with c-myb antisense may lead to decreased fibrosis in wound healing and may lead to novel treatments of diseases like scleroderma, improving wound healing and decreasing scar formation.

Conclusion

It is now well documented that c-myb is a transcription factor involved in regulating the balance between cell division, differentiation and survival. Excessive deposition of type I collagen is highly characteristic of numerous fibrotic disorders including scleroderma and hypertrophic scars. Numerous researchers have speculated that excessive deposition of type I collagen seen in these disorders most likely takes place due to high transcriptional activation of collagen genes in response to various cytokines like TGF-ß and other factors present in prefibrotic inflammatory lesions, for example c-myb protein [4, 15-17]. Current data strongly suggest that c-myb is an important regulator of type I collagen and plays an important role in controlling the proliferation of fibroblasts.

Existing treatment options aimed at reducing collagen levels in hypertrophic scars, including surgery, biophysical therapeutics and pharmacological therapies, are highly unspecific and their outcomes unpredictable. Our results have shown that c-myb is significantly increased in both epidermal keratinocytes and dermal fibroblasts in hypertrophic scar tissue. As such, future research aimed at understanding

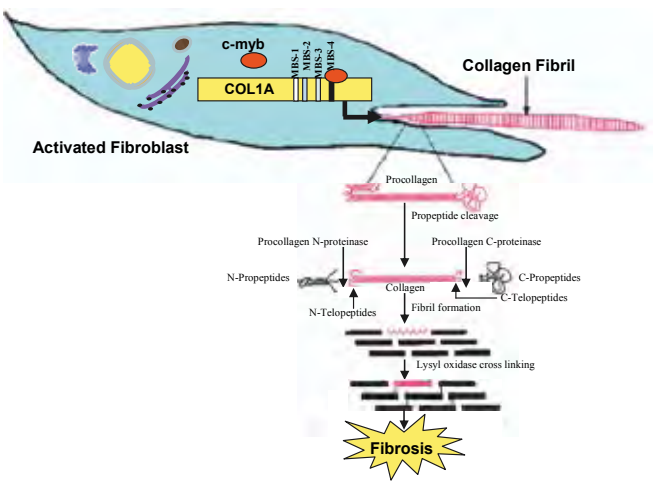

Figure 3. c-myb modulation of collagen expression regulates dermal fibrosis levels. Schematic representation of c-myb-binding to the myb-binding sites (MBS) on the collagen type I promoter (COL1A2) in fibroblasts that leads to increased collagen type I expression and cutaneous fibrosis.

the mechanisms by which c-myb regulates type I collagen production may lead to novel mechanistic-based therapeutic options aimed at reducing fibrosis during wound healing process and a better comprehension of many fibrotic diseases like scleroderma.

Acknowledgements

AJ Cowin is supported by a fellowship from the NHMRC and funding from Channel 7 Children's Research Foundation.

References

1. Diegelmann RF & Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci 2004; **9**:283-9.
2. Desmouliere A, Chaponnier C & Gabbiani G. Tissue repair, contraction, and the myofibroblast. Wound Repair Regen 2005; **13**:7-12.
3. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C & Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodeling. Nat Rev Mol Cell Biol 2002; **3**:349-63.
4. Piccinini G, Golay J, Flora A, Songia S, Luchetti M, Gabrielli A & Introna M. C-myb, but not B-myb, up-regulates type I collagen gene expression in human fibroblasts. J Invest Dermatol 1999; **112**:191-6.
5. Kopecki Z, Luchetti MM, Adams DH *et al.* Collagen loss and impaired wound healing is associated with c-Myb deficiency. J Pathol 2006; **211**:351-61.
6. Ramsay RG, Barton AL & Gonda TJ. Targeting c-Myb expression in human disease. Expert Opin Ther Targets 2003; **7**:235-248.
7. Golay J, Capucci A, Arsura M, Castellano M, Rizzo V & Introna M. Expression of c-myb and B-myb, but not A-myb, correlates with proliferation in human hematopoietic cells. Blood 1991; **77**:149-58.
8. Miglarese MR, Richardson AF, Aziz N & Bender TP. Differential regulation of c-Myb-induced transcription activation by a phosphorylation site in the negative regulatory domain. J Biol Chem 1996; **271**:22697-705.
9. Piccinini G, Luchetti MM, Caniglia ML, Carossino AM, Montroni M, Introna M & Gabrielli A. c-myb proto-oncogene is expressed by quiescent scleroderma fibroblasts and, unlike B-myb gene, does not correlate with proliferation. J Invest Dermatol 1996; **106**:1281-6.
10. Xu H, Inouye M, Hines E, Collins J & Ghishan F. Transcriptional regulation of the human NaPi-IIb co-transporter by EGF in Caco-2 cells involves c-myb. Am J Physiol 2003; **284**:1262-71.
11. Nordgard O, Andersen TO & Gabrielsen OS. Selective inhibition of c-Myb DNA-binding by RNA polymers. BioMed Central Biochem 2004; **5**:15.
12. Nomura T, Sakai N, Sarai A *et al.* Negative autoregulation of c-Myb activity by homodimer formation through the leucine zipper. J Biol Chem 1993; **268**:21914-23.
13. Luchetti MM, Paroncini P, Majlingova P *et al.* Characterization of the c-Myb-responsive region and regulation of the human type I collagen alpha 2 chain gene by c-Myb. J Biol Chem 2003; **278**:1533-41.
14. Ma HH, Yao JL, Li G, Yao CL, Chen XJ & Yang SJ. Effects of c-myb antisense RNA on TGF-beta1 and beta1-I collagen expression in cultured hepatic stellate cells. World J Gastroenterol 2004; **10**:3662-5.
15. Ihn H, Ohnishi K, Tamaki T, LeRoy EH & Trojanowska M. Transcriptional regulation of the human alpha 2(I) collagen gene. Combined action of upstream stimulatory and inhibitory cis-acting elements. J Biol Chem 1996; **271**:26717-23.
16. Ihn H & Trojanowska M. Sp3 is a transcriptional activator of human alpha2(I) collagen gene. Nucleic Acids Res 1997; **25**:3712-7.
17. Gosh AK. Factors involved in the regulation of type I collagen gene expression: implications in fibrosis. Exp Biol Med 2002; **227**:301-14.