Androgens modulate the inflammatory response during acute wound healing

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Summary

Impaired wound healing states in the elderly lead to substantial morbidity and mortality, and a cost to the health services of over $9 billion per annum. In addition to intrinsic ageing processes that per se cause delayed healing, studies have suggested marked differences in wound repair between the sexes. We have previously reported that, castration of male mice results in a striking acceleration of local cutaneous wound healing and dampens the associated inflammatory response. In this study, we report that systemic 5α-reductase inhibition, which blocks the conversion of testosterone to its more active metabolite 5α-dihydrotestosterone, mimics the effects of castration in a rat model of cutaneous wound healing. The mechanisms underlying the observed effects involve a direct, cell-specific upregulation of pro-inflammatory cytokine expression by macrophages, but not fibroblasts, in response to androgens. Androgens require the transforming growth factor β signalling intermediate Smad3 to be present in order to influence repair and local pro-inflammatory cytokine levels. That reducing 5α-dihydrotestosterone levels through 5α-reductase antagonism markedly accelerates healing suggests a specific target for future therapeutic intervention in impaired wound healing states in elderly males.

Key words: Wound healing, Inflammation, DHT, Testosterone

Introduction

Cutaneous wound healing is a complex process that is vitally important in restoring the dermal barrier and preventing infection following injury. However, repair does not always proceed smoothly. Hypertrophic scars and keloids result from excessive matrix deposition whereas venous insufficiency and diabetes mellitus often result in the formation of chronic, non-healing ulcers. The treatment of such aberrant wound healing conditions places a significant financial burden upon health services on a global scale. Elderly males have been observed to heal wounds more slowly than females, and with reduced matrix deposition and an enhanced inflammatory response (Ashcroft et al., 1999a; Ashcroft and Mills, 2002). These, and additional gender-related distinctions in wound healing parameters, prompted the suggestion that circulating sex hormones are key modulators of the response to cutaneous injury. Initial studies showed that estrogens accelerate wound repair in both human and rodent models by dampening local inflammation (Ashcroft et al., 1997a; Ashcroft et al., 1999a). More recent evidence has suggested that androgens are negative regulators of the healing process. Raised serum testosterone levels were found to be inversely correlated with the rate of healing of acute wounds in elderly health-status-defined human males (Ashcroft and Mills, 2002). In a murine wound healing model, repair progressed more rapidly in castrated animals compared with controls (Ashcroft and Mills, 2002). This response was accompanied by decreased wound inflammatory cell influx and elevated matrix collagen deposition. Similarly, systemic treatment with the androgen receptor (AR) antagonist flutamide accelerated healing and depressed the inflammatory response (Ashcroft and Mills, 2002).

The roles of individual androgenic species in regulating wound repair are yet to be determined, although the immunolocalisation of AR to keratinocytes, inflammatory cells and fibroblasts suggests that they are involved in the regulation of inflammation and/or repair (Ashcroft and Mills, 2002). The primary 'male' sex steroid testosterone is converted to its more active metabolite 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase (5α-R), of which two isoforms exist (5α-R1 and 5α-R2); both are expressed by hair follicles and the epidermis in uninjured skin (Eicheler et al., 1995; Thiboutot et al., 2000). Several studies have demonstrated cell-specific and differential regulation by testosterone and DHT of proteins with key roles in cutaneous repair. Testosterone was found to inhibit production of interleukin 1 (IL-1) by murine macrophages and of IL-6 by human peripheral blood monocytes (Savita and Rai, 1998; Kanda et al., 1996) but to increase chondrocyte IL-6 secretion and macrophage tumour neurosis factor α (TNF-α) generation, in the latter case acting at the AR (Ashcroft and Mills, 2002; Guerne et al., 1990). It is not known, however, whether these responses result from the conversion of testosterone to DHT, which itself reduces IL-6 expression in osteoblasts and stimulates transforming growth factor β1 (TGF-β1) secretion by prostate cancer LNCaP cells (Hofbauer et al., 1999; Blanchère et al., 2002). DHT treatment has also been found to decrease matrix metalloproteinase 1 (MMP1) mRNA levels in LNCaP cells (Schneikert et al., 1996); to enhance expression of procollagen I in bone and skin and of matrix metalloproteinase 2 (MMP2) and procollagens I, III and IV in adipose tissue (Benz et al., 1991; Opas et al., 2000; Davey et al., 2000; Bolduc et al., 2004); and to stimulate the proliferation of cultured human prostate keratinocytes and

promote their expression of the pro-angiogenic vascular endothelial growth factor (VEGF) (Sordello et al., 1998; Planz et al., 2001).

Although such findings provide insights into the mechanisms by which androgens influence cellular physiology, the roles of specific androgenic species in the in vivo regulation of wound healing have never before been directly studied. To this end, we have utilised a rodent wound healing model to investigate the effect that blocking the conversion of testosterone to DHT has upon dermal repair. Male Sprague-Dawley rats treated with the dual-specificity 5α-R inhibitor MK-434 exhibit accelerated wound repair and reduced local inflammation. This response bears strong similarities to the situation in castrated animals in which the production of both DHT and testosterone is blocked, suggesting that testosterone acts to inhibit wound healing through conversion to DHT. Additional studies employing a similar model in Smad3-null ($Smad3^{-/-}$) mice have identified a potential role for the TGF-β-activated transcription factor and AR-interacting protein Smad3 in mediating the pro-inflammatory actions of androgens. Taken together, our data suggest that the inhibition of DHT production, systemically or locally, could be a potential therapeutic target for the acceleration of healing in elderly males.

Results

5α-reductase inhibition accelerates wound healing and modulates the local inflammatory response

From previous studies in control and castrated mice and rats, gross macroscopic measurements of wounds immediately following incision exhibit no statistical differences in gape or area. However, following full-thickness dorsal incisional wounding of castrated male rats and intact littermates, an accelerated rate of wound healing was readily apparent in the androgen-deficient animals at days 2 and 6 post-wounding, with wound areas significantly reduced at day 2 (Fig. 1A,C). Systemic treatment with MK-434 at the time of wounding elicited a greater than threefold reduction in circulating DHT levels (our unpublished observation) and provoked a significant decrease in wound areas, paralleling the effects of castration. Not only were wounds smaller in the androgen-deficient mice, and in those in which DHT biosynthesis was blocked, but there was also a substantial decrease in the numbers of inflammatory cells per unit area. Immunolocalisation of specific neutrophil

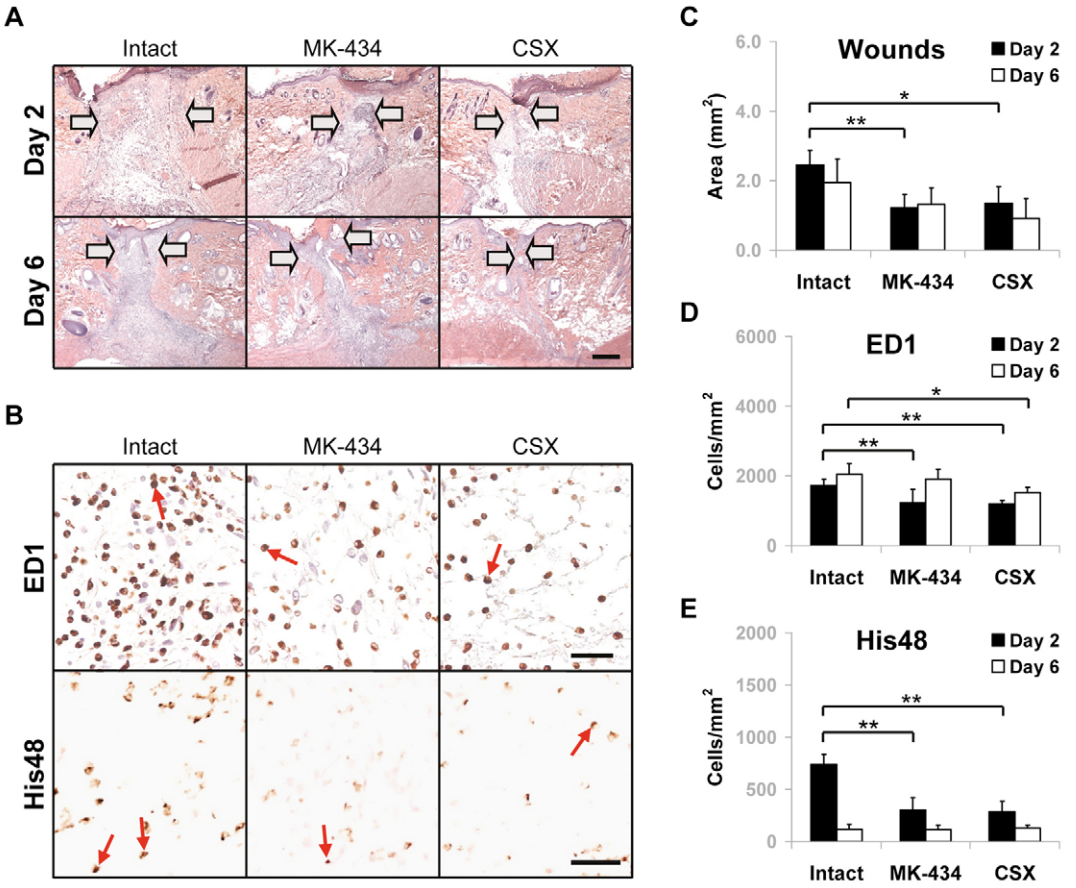

Fig. 1. In rats, incisional wound healing is accelerated and inflammatory cell influx attenuated by 5α-reductase inhibition. (A,C) Day-2 and -6 wound sections were subjected to histological analysis and quantification. Wound areas at day 2 post wounding were reduced in wounds of castrated (CSX) animals and those treated with MK-434 (n=5-7 per group). Arrows indicate the wound margins. (B,D) Numbers of ED1-positive macrophages (studied immunohistochemically, quantified and indicated by arrows) in day-2 and -6 wounds of castrated animals were reduced as they were in day-2-wounds of animals treated with MK-434. (B,E) Numbers of His48-immunoreactive granulocytes (arrows) were significantly reduced both in day-2-wounds of castrated animals and those treated with MK-434. Bars, 500 µm (A) or 50 µm (B). Data represent mean ± s.d. *P<0.05; **P<0.01.

and macrophage markers indicated that populations of both cell types were significantly reduced in the wounds of castrated and MK-434-treated animals compared with intact controls (Fig. 1B,D,E). Hence, systemically blocking DHT production accelerates healing and dampens local inflammation. AR staining patterns were qualitatively and quantitatively similar between the animal treatment groups (our unpublished observation), suggesting that altered AR expression did not contribute to the observed responses.

Androgens modulate local production of specific cytokines in wound tissue

In a number of patho-physiological tissue processes, increased inflammation can be attributed to altered levels of IL-6, TGF-β1 and TNF-α (Brennan et al., 1989; Mahida et al., 1991; Cheon et al., 2002; Pooran et al., 2003). To delineate further the actions of androgens on tissue-repair responses, we investigated whether castration or treatment with MK-434 impacted upon the levels of these specific inflammatory cytokines within the local milieu of incisional wounds. All three cytokines were immunolocalised to both epidermal keratinocytes and cells displaying morphological characteristics of macrophages, neutrophils and fibroblasts.

The pro-inflammatory cytokine IL-6 is a key player in the wound-repair process as evidenced by the observation that healing is impaired in IL-6 knockout mice (Lin et al., 2003). IL-6 was immunolocalised to inflammatory cells and the proliferating epidermis of day 2 rat wounds (Fig. 2A). Castration and MK-434 treatment induced a significant decrease in day-2-wound IL-6-immunopositive cell numbers and, in the case of castration, mRNA expression (Fig. 2A-C). Immunoblotting corroborated the results obtained from immunostaining and cell quantitation (Fig. 2D). TNF-α and TGF-β1 are two further cytokines with important wound healing roles, both of which were highly expressed by inflammatory cells in day 2 rat wounds (Fig. 2A). TNF-α is a regulator of angiogenesis, inflammation and fibroplasia during repair (Garlick et al., 1994; Huttunen et al., 2000). Castration, but not MK-434 treatment, reduced day-2-wound TNF-α-positive cell numbers, protein levels and mRNA expression (Fig. 2B-D). TGF-β1 delays re-epithelialisation but stimulates wound contraction and is a crucial promoter of wound matrix

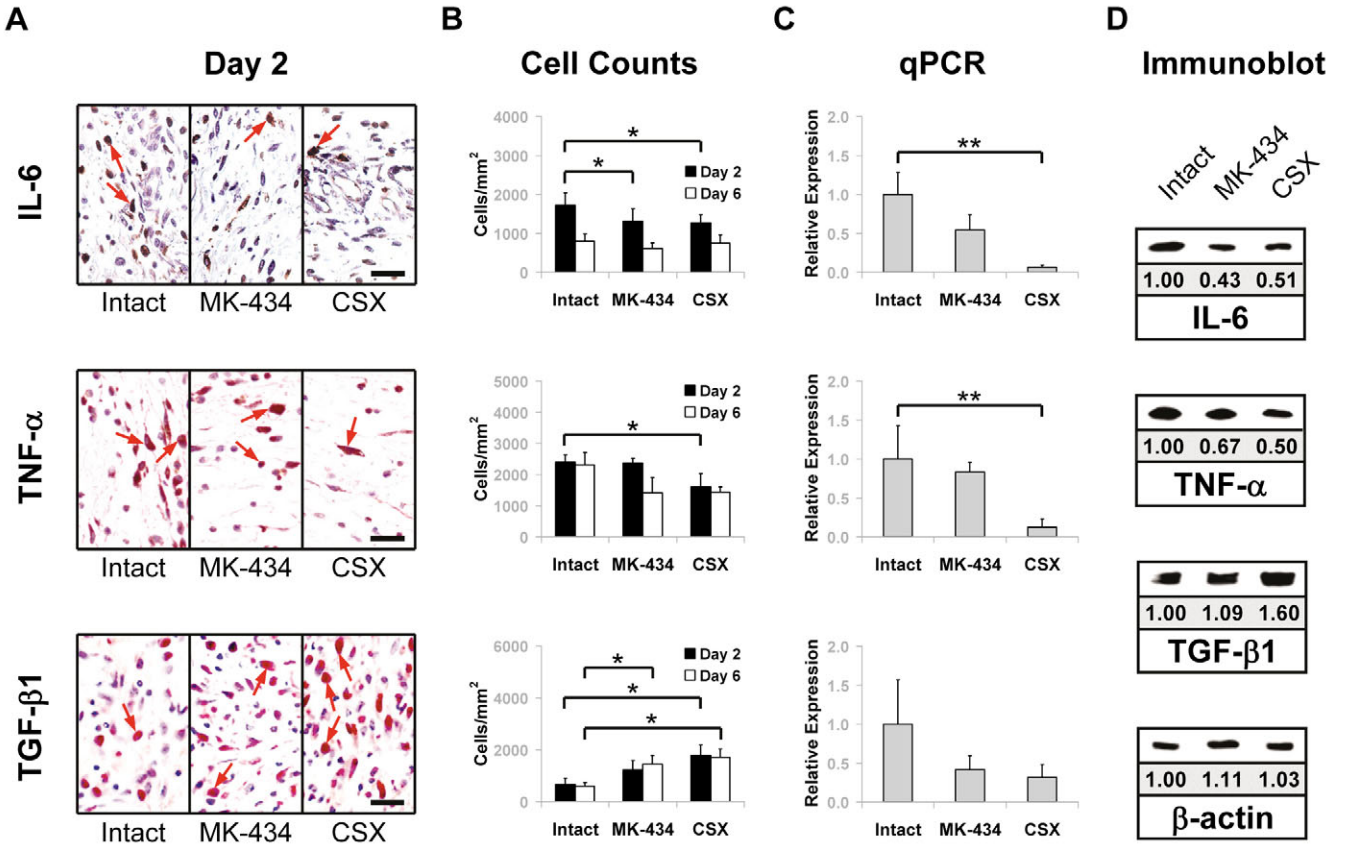

Fig. 2. Wound inflammatory cytokine mRNA and protein levels are modulated by MK-434 treatment and castration. (A-D) Incisional day-2-wound sections were analysed immunohistochemically and tissue mRNA and protein extracts were analysed by qPCR and immunoblotting, respectively (n=6-7 per treatment group). Day 6 wound sections were analysed immunohistochemically. Arrows indicate immunoreactive cells. Numbers of IL-6-immunopositive cells, overall protein levels and gene expression were reduced in day-2-wounds of castrated animals and those treated with MK-434 (gene expression in castrated animals only). Numbers of TNF-α-immunoreactive cells, levels of TNF-α protein and mRNA were also reduced in day-2-wounds of castrated animals. Numbers of TGF-β1-immunopositive cells and TGF-β1 protein levels were increased in day-2-wounds of castrated rats; also increased were numbers of TGF-β1-immunopositive cells in day-6-wounds of castrated animals and of those treated with MK-434. Numbers in D represent relative levels of protein based on optical density readings, normalised to the β-actin signal. Bars, 20 μm. Data represent mean ± s.d. *P<0.05; **P<0.01.

protein deposition (Nakao et al., 2003; Park et al., 2000; Roberts et al., 1986). In the current study we found that castration significantly increased day-2-wound TGF-β1-immunoreactive inflammatory cell numbers and overall wound TGF-β1 protein levels, whereas both castration and MK-434 treatment increased TGF-β1-immunopositive cell numbers in day 6 wounds (Fig. 2B,D). By contrast, day-2-wound TGF-β1 mRNA levels tended to be reduced by castration and MK-434 treatment (Fig. 2C) suggesting that androgens inhibit the production or persistence of TGF-β1 message but positively regulate post-transcriptional events such as message translation or protein stabilisation. The reductions in TNF-α and IL-6 mRNA levels in wounds from castrated rats might result from either (1) a decrease in the population of mRNA-expressing cells or (2) a decrease in the amount of message produced per cell.

Viewed together, these data suggest that androgens influence healing progression by modulating the acute inflammatory response and by altering the dynamic balance of wound cytokine levels. Moreover, the conversion of testosterone to DHT is required to effect a proportion of the observed responses. IL-6 is identified as being a putative DHT-induced factor, whereas wound TNF-α and TGF-β1 levels appear to be regulated also by testosterone. Intriguingly, 5α-R1 and 5α-R2 were expressed by only a small proportion of wound inflammatory cells (our unpublished observations). This implies that it is the blockade of gonadal, rather than local, DHT production that is largely responsible for effecting these responses, although it should be noticed, that DHT synthesised from testosterone by epidermal keratinocytes may have significant paracrine effects upon proximal inflammatory cells.

Regulation of gene expression by androgens is cell-specific

Having determined that blocking DHT production enhances wound healing and modifies inflammatory cytokine levels in the context of a rat in vivo model, we next investigated the effects of in vitro androgen treatment on two of the fundamentally important cell types associated with dermal repair: macrophages and fibroblasts. Since the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI 3-kinase) pathways have previously been implicated in androgen signalling (Kang et al., 2004; Peterziel et al., 1999), the mechanisms underpinning the responses to DHT were further studied by co-treating with a range of inhibitory substances including the MAPK pathway blocker PD 98059 and the PI 3-kinase signalling inhibitor wortmannin.

Treatment of peritoneal macrophages with DHT and testosterone induced a significant increase in expression of the *IL6* gene while having no discernable effect upon TNF-α and TGF-β1 mRNA levels (Fig. 3A). That TGF-β1 mRNA levels were unaffected by actinomycin D suggests that the turnover of this mRNA species occurs on a longer time scale than that tested. The IL-6 response to DHT was blocked by actinomycin D, suggesting the involvement of a transcriptional mechanism, and by PD 98059 and wortmannin, implicating the MAPK and PI 3-kinase pathways in the induction of IL-6 expression. By contrast, treatment with DHT significantly reduced the expression of IL-6, TNF-α and TGF-β1 mRNAs in dermal fibroblasts (Fig. 3B). Furthermore, secretion of IL-6 and total (active and inactive) TGF-β1, as assessed by ELISA, was significantly depressed in response to DHT (Fig. 3C). However, the DHT-elicited reduction in TNF-α mRNA expression was not paralleled by a similar decrease in the levels of protein (our unpublished observation), suggesting an alteration in translational efficiency or protein stability. Treatment with PD 98059 blocked the DHT-induced attenuation of TNF-α mRNA expression, indicating the possible involvement of MAPK signalling in mediating this response. Furthermore, wortmannin reversed the inhibitory effect of DHT on the expression of IL-6, TNF-α and TGF-β1 mRNAs, suggesting a crucial contribution of the PI 3-kinase pathway to the downregulation of inflammatory cytokine gene expression in fibroblasts. Intriguingly, co-treatment with the translational inhibitor cycloheximide alone or in combination with DHT (data not shown), provoked a significant rise (> tenfold) in the level of IL-6 mRNA and a dramatic increase (>1000-fold) in TNF-α mRNA levels (Fig. 3D,E). A similar superinduction of the mRNAs encoding IL-6- and TNF-α has previously been documented in other cellular contexts (Osipovich et al., 1993; Hershko et al., 2004). TGF-β1 mRNA expression was not, by contrast, significantly modulated by cycloheximide (Fig. 3F). These observations suggest that the expression by dermal fibroblasts of genes encoding certain inflammatory cytokines is subject to strict negative control by one or more unidentified proteins. Based on these collective observations, androgens appear to have broadly distinct effects upon cytokine expression in fibroblasts and macrophages, with macrophage responses more closely correlating with the pattern in wound tissue.

Androgens act though Smad3 to modulate cytokine levels in wound tissue

Smad3 is a TGF-β-activated transcription factor for which important roles in mediating a number of wound repair-associated TGF-β1 responses have been documented, including the stimulation of monocyte chemotaxis and matrix deposition (Ashcroft et al., 1999b). More recently, Smad3 has been shown to inhibit, through direct interaction, AR transactivation in human prostate cancer cells, whereas, conversely, AR has been observed to enhance and inhibit Smad3 signalling (Hayes et al., 2001; Kang et al., 2001; Chipuk et al., 2001). Such in vitro observed cross-talk might additionally relate to specific situations in vivo, in which Smad3 and sex steroids have been jointly implicated.

We have previously shown that, whereas castration reduces areas and cell numbers in 3-day-old wounds of wild-type mice, it has no effect on these parameters in Smad3$^{-/-}$ animals, suggesting that Smad3 is a critical factor mediating the effects of androgens on wound healing (Ashcroft et al., 2003a). To investigate the potential roles of Smad3 in linking androgen signalling to wound-associated inflammation, we have studied the effects of castration and androgen replacement on wound inflammatory cell numbers and cytokine levels in wild-type and Smad3$^{-/-}$ mice.

Wound macrophage numbers were significantly reduced by castration of wild-type mice (Fig. 4A). By contrast, castration did not significantly modulate numbers of macrophages in the wounds of Smad3$^{-/-}$ mice. Similarly, wound neutrophil numbers in castrated animals were reduced compared with sham-operated wild-type control mice but not Smad3$^{-/-}$ mice (Fig. 4B). When the effects of manipulating androgen levels on wound inflammatory cytokine levels were studied, a similar

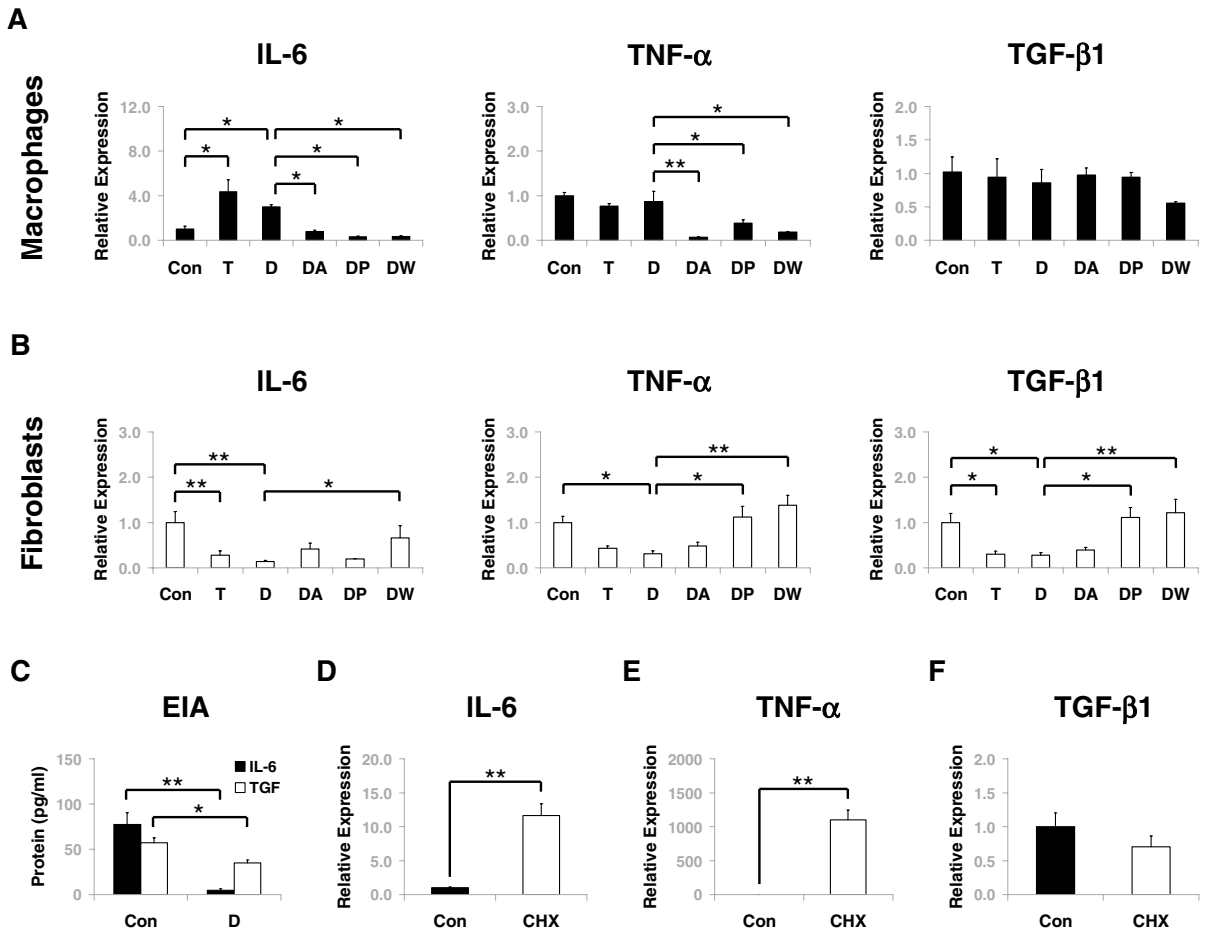

Fig. 3. mRNA levels of inflammatory cytokines in macrophages are attenuated by MAP kinase and PI 3-kinase inhibitors, which additionally block the inhibition of fibroblast cytokine mRNA expression by DHT. Macrophage- and fibroblast-derived mRNA samples were analysed by qPCR and supernatants by ELISA. (A) The increase in macrophage IL-6 mRNA levels in response to DHT was blocked by actinomycin D, PD 98059 and wortmannin. By contrast, TNF-α mRNA expression was unaffected by treatment with DHT or testosterone but was attenuated by actinomycin D, PD 98059 and wortmannin. Treatment with wortmannin tended to reduce TGF-β1 mRNA levels that were otherwise unaffected by androgen treatment. (B) In fibroblasts, the decrease in IL-6 mRNA levels in response to DHT was blocked by wortmannin but not PD 98059. Expression of TNF-α mRNA was similarly attenuated by DHT, a response that was blocked by treatment with PD 98059 or wortmannin. Co-treatment with PD 98059 or wortmannin reversed the inhibitory effect of DHT on fibroblast TGF-β1 mRNA expression. (C) Fibroblast secretion of IL-6 and total TGF-β1 protein levels (active and inactive) was reduced after treatment with DHT. (D-F) Fibroblast mRNA levels of IL-6 and TNF-α were dramatically increased following treatment with cycloheximide. Fibroblast TGF-β1 mRNA levels were unaffected. Data represent mean ± s.d. *P<0.05; **P<0.01. Con, control; T, testosterone; D, DHT; A, actinomycin D; P, PD 98059; W, wortmannin; CHX, cycloheximide.

pattern emerged: TNF-α-positive cell numbers were significantly reduced by castration of wild-type animals but were unaffected in Smad3$^{-/-}$ mice (Fig. 4C). Similarly, castration increased TGF-β1-immunostained cell numbers in wild-type animals only (Fig. 4D). Given the crucial contribution Smad3 makes to TGF-β1 autoinduction (Piek et al., 2001), the possibility that TGF-β1 levels are generally not inducible in Smad3$^{-/-}$ mice cannot be excluded. Nevertheless, the current findings provide further evidence that Smad3 is a potentially important mediator of androgens' effects on wound healing and local inflammation.

Smad-signalling intermediates are modulated in vivo and in vitro by androgens

Having identified Smad3 as a putative factor required by androgens to modulate wound-associated inflammation, we next studied the effects of castration on the expression of the regulatory Smad3, its DNA binding partner Smad and the TGF-β-signalling inhibitor Smad7. Immunohistochemical analysis of day 2 rat wounds (Fig. 5A,B) showed that wound Smad3- and Smad4-positive cell numbers were unaffected by castration or MK-434 treatment, although Smad4-positive cell numbers were reduced in the day 6 wounds of MK-434-treated and castrated animals compared with controls (our unpublished observation). Day-2-wound levels of phospho-Smad3 (Fig. 5A) were similar between control, MK-434-treated and castrated rats, which suggests that neither DHT nor testosterone significantly modulates overall wound Smad3 activation rates. By contrast, day-2-wound Smad7-positive cell numbers (Fig. 5A,B) were significantly increased by castration and MK-434 treatment, suggesting that DHT reduces overall Smad7 protein levels within the wound. This response was not,

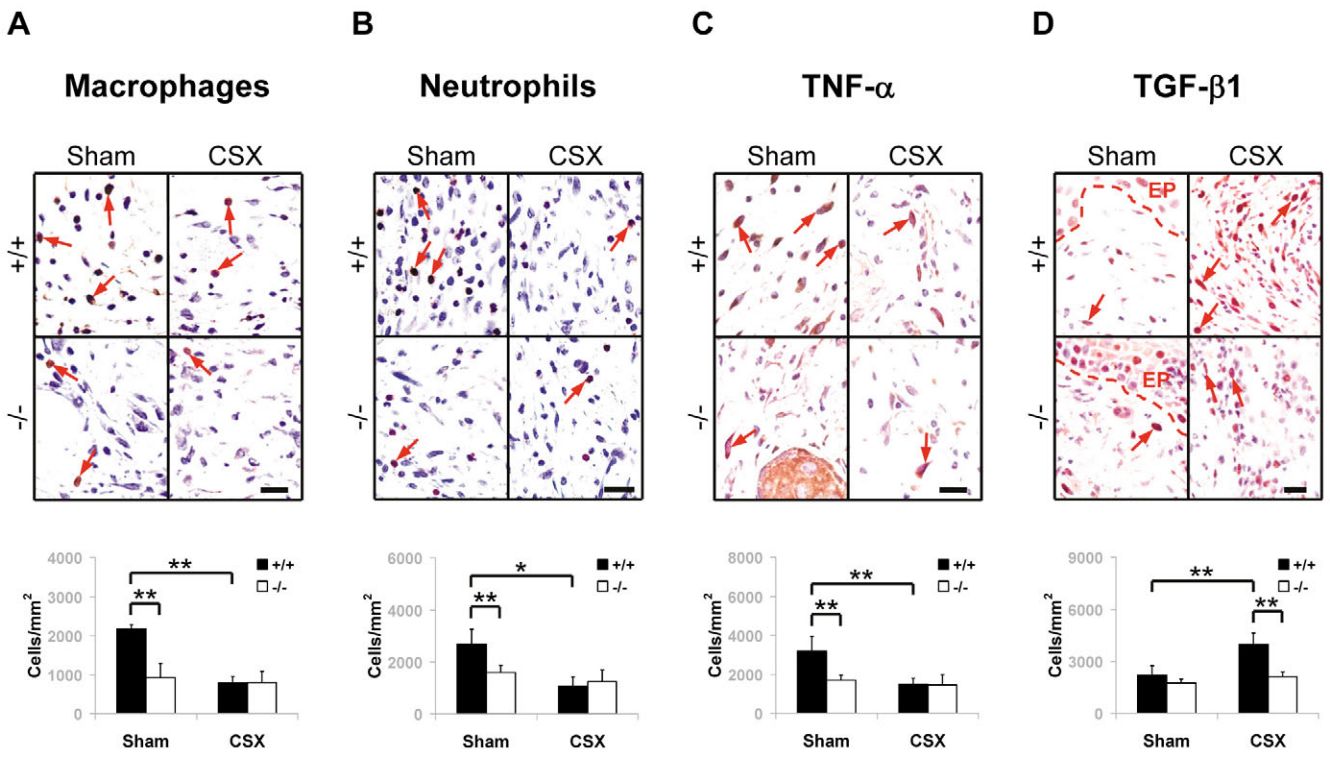

Fig. 4. Numbers of macrophages, neutrophils and TNF-α-positive cells in wounds of Smad3$^{-/-}$ mice are not affected by castration. Day 3 wound sections were analysed immunohistochemically to quantify inflammatory cells, and IL-6- and TNF-α-immunoreactive cell numbers. (A) Numbers of macrophages were significantly reduced in day-3-wounds of castrated wild-type mice but were not affected in day-3-wounds of Smad3$^{-/-}$ animals (n=4-5 per treatment group). (B) The population of neutrophils was similarly depleted in wounds of castrated wild-type mice but not of Smad3$^{-/-}$ animals. (C) Numbers of TNF-α-positive cells were decreased in day-3-wounds of castrated wild-type animals but were not affected in day-3-wounds of castrated Smad3$^{-/-}$ mice. (D) Numbers of TGF-β1-immunoreactive inflammatory cells were increased solely in wounds of castrated wild-type mice. Arrows indicate immunoreactive cells; dashed lines in D indicate the dermal-epidermal boundary. Bars, 20 μm. Data represent mean ± s.d. *P<0.05; **P<0.01. EP, epidermis.

however, mirrored at the message level: castration significantly reduced Smad4 and Smad7 mRNA expression in day 2 wounds (Fig. 5C). All three Smad proteins studied were localised to the proliferating epidermis and cells displaying morphological characteristics of macrophages, neutrophils and fibroblasts. Smad7 immunostaining was also localised to endothelial cells, which suggests that it may be involved in any potential direct effects of TGF-β on wound angiogenesis. Furthermore, endothelial cell staining intensity appeared to be especially intense in wounds from castrated animals (Fig. 5A).

TGF-β1 and Smad intermediates showed a ubiquitous pattern of immunostaining, present in all major cell types involved in healing and particularly prominent in inflammatory cells. Our previous findings have demonstrated that macrophages are important cellular targets for androgen action during wound healing, whose local populations and inflammatory cytokine production are reduced by castration and MK-434 treatment. We therefore investigated the effects of in vitro androgen treatment on macrophage Smad gene expression (Fig. 5D). Exposure to testosterone or DHT triggered a significant increase in Smad3 mRNA levels. Additionally, DHT but not testosterone significantly increased expression of Smad7 mRNA and tended to increase Smad4 mRNA levels, suggesting that, whereas both testosterone and DHT are potent inducers of macrophage Smad3 expression, only DHT is capable of eliciting an alteration in the expression of the other Smad genes. Moreover, basal gene expression appeared to be repressed, in the case of Smad3, by activation of the PI 3-kinase pathway and, for Smad7, by MAPK signalling (our unpublished observations). Overall, these data provide evidence that DHT might, during wound healing, influence TGF-β signalling pathways by modulating the relative ratios of regulatory and inhibitory Smad intermediates (Fig. 6).

Discussion

Differences in circulating sex steroid levels may underscore gender- and age-related disparities in wound healing progression. Data from both human and rodent models indicate that estrogens accelerate wound healing and dampen the local inflammatory response (Ashcroft et al., 1997a; Ashcroft et al., 1999a; Ashcroft et al., 2003b). The regulatory roles of androgens are less well characterised. Reports suggest that healing of incisional wounds is accelerated in castrated mice compared with controls, and is accompanied by reduced inflammation and an increase in wound collagen content (Ashcroft and Mills, 2002). To expand our understanding in this area, we employed a rat model of incisional wound healing to investigate with the 5α-reductase inhibitor MK-434 the impact of blocking the conversion of testosterone to DHT. By comparing the impact of the inhibitor with the effects of castration (which abrogates the biosynthesis of both DHT and

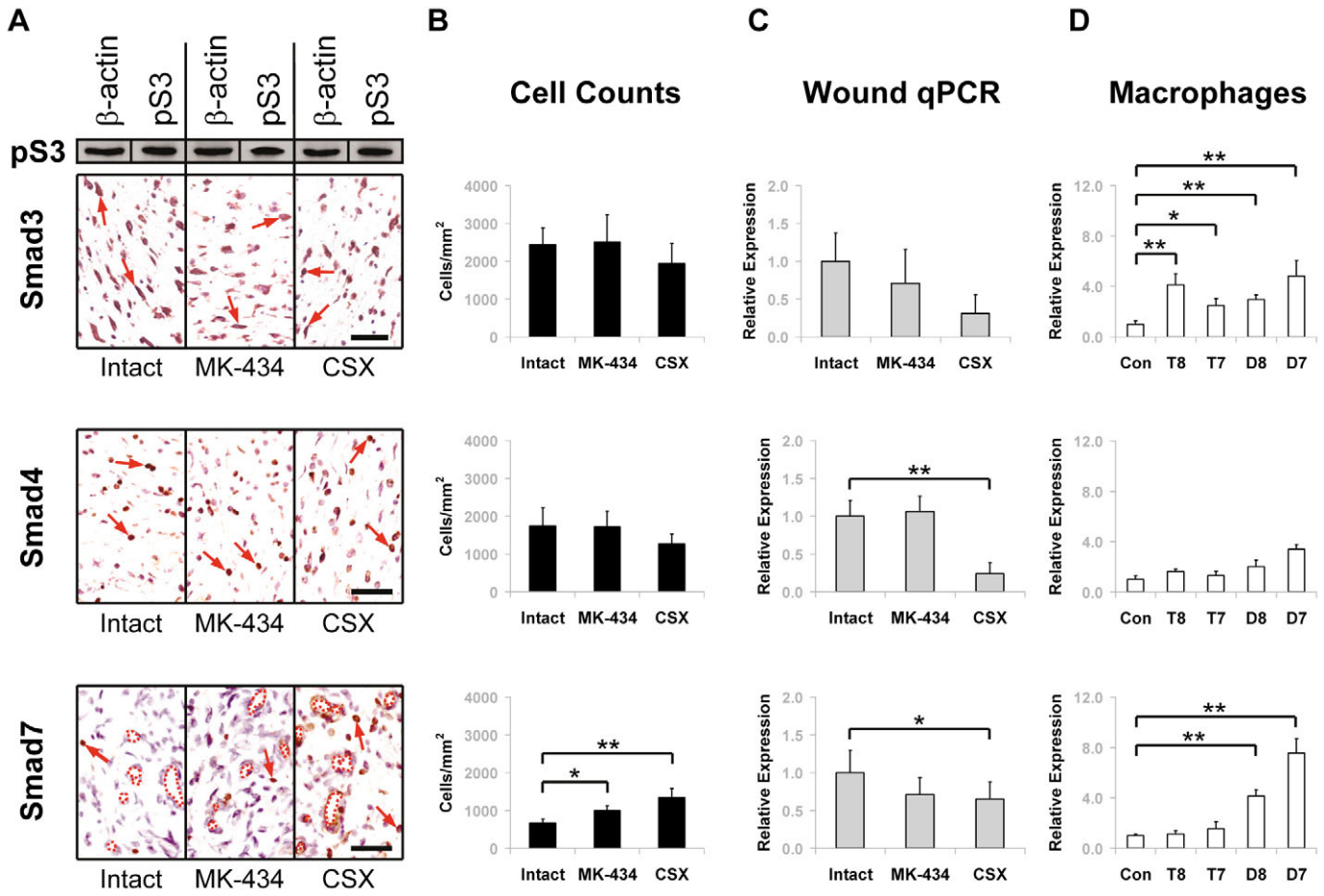

Fig. 5. Smads are regulated by androgens and castration. Day-2-wound sections were analysed immunohistochemically, wound protein samples by western blotting, and tissue and macrophage-derived mRNA samples by qPCR. (A-B) Numbers of Smad3- and Smad4-immunopositive cells and protein levels of phosphorylated (activated) Smad3 (pS3) were unaffected in wounds of castrated animals and those treated with MK-434. Smad7-positive cell numbers were significantly increased in day-2-wounds of castrated animals and those treated with MK-434. (C) Castration of animals reduced Smad4 and Smad7 mRNA levels in wounds. (D) Treatment of peritoneal macrophages with testosterone or DHT for 18 hours significantly increased the levels of Smad3 mRNA, whereas exposure to DHT but not testosterone increased mean Smad4 mRNA levels and significantly increased Smad7 mRNA levels. Arrows indicate immunostained cells; dashed lines surround blood vessels. Bars, 50 μm. Data represent mean ± s.d. **P*<0.05; ***P*<0.01. Con, control; T7 and T8, 10^{-7} M and 10^{-8} M testosterone, respectively; D7 and D8, 10^{-7} M and 10^{-8} M DHT, respectively.

testosterone) it was possible to determine which of the observed responses are specific to DHT and which are shared with testosterone. Castration of male rats resulted in a reduction of wound areas and inflammatory cell counts, mirroring previous findings in mice (Ashcroft and Mills, 2002). MK-434 treatment similarly resulted in a significant reduction in day-2-wound areas and inflammatory cell populations. Incisional wound areas in rodents have previously been inversely correlated with wound breaking strength (Ashcroft et al., 2003b), which suggests that the biomechanical properties of healing wounds are improved following MK-434 treatment or castration. Moreover, inhibiting DHT formation selectively reduced and increased in vivo wound levels of IL-6 and Smad7 respectively, implicating these factors in the repair responses to DHT. That MK-434 has a positive effect on these fundamental wound healing parameters strongly suggests that conversion to DHT is crucial for testosterone to be able to exert a negative influence on the repair process. In this context, it is also noteworthy that both 5α-reductase isoforms were strongly expressed in venous ulcer biopsy tissue from human males, in whom raised circulating DHT levels were associated with an increased incidence of such ulceration (our unpublished observations). Connections between androgen status and ulceration rates are not unprecedented. Indeed, recent neural network studies have confirmed that the primary risk factor for ulceration is a male genotype (Taylor et al., 2002). The specific androgen mediating these effects, and its mechanisms of action, are unknown, although our observations suggest that DHT contributes to the underlying pathology.

An excessive or aberrant inflammatory response is well-recognised as a major contributing factor to delayed healing in both animal and human models. The mechanisms underpinning the reductions in wound macrophage and neutrophil numbers in this study might derive from altered pro-inflammatory cytokine expression, dysregulated adhesion molecule profiles or direct effects on cell chemotaxis. DHT has previously been found to increase surface expression of the macrophage- and neutrophil-binding adhesion molecule VCAM-1 in human

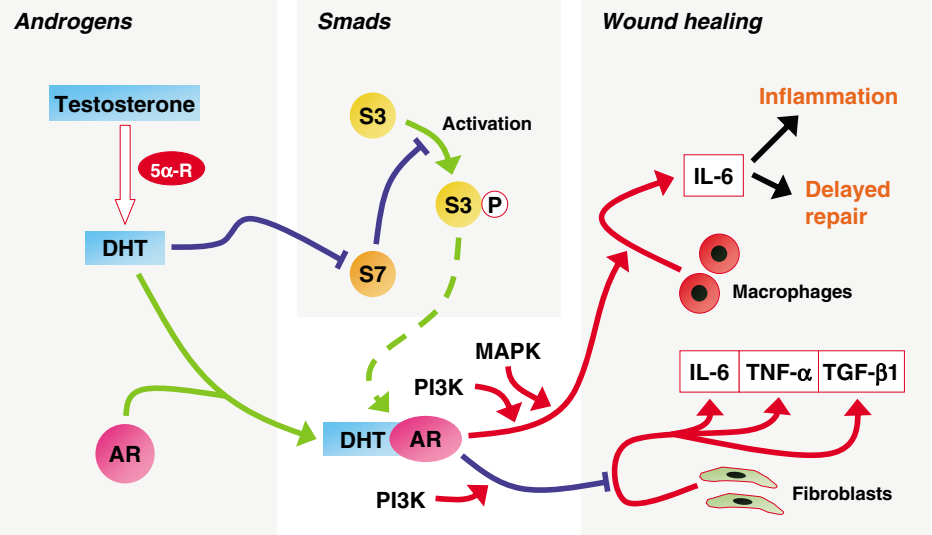

Fig. 6. Proposed mechanism for the androgenic regulation of wound healing. DHT binds to the AR and the resulting ligand-activated AR represents an effector complex whose activities may be modulated by Smad3. Activated AR stimulates macrophage production of pro-inflammatory cytokines, while at the same time depressing fibroblast cytokine expression and secretion. The increase in macrophage cytokine release, which depends on MAPK and PI 3-kinase signalling, contributes to enhanced inflammation and hence delayed healing.

TNF-α-activated umbilical vein endothelial cells, resulting in increased monocyte adhesion in an in vitro co-culture system (McCrohon et al., 1999). Alternatively, androgens might increase wound inflammatory cell numbers by stimulating proliferation or chemotaxis, although a previous study failed to show a chemotactic response to DHT and testosterone in macrophages (Ashcroft and Mills, 2002). It is worth noticing that macrophage activation and subpopulation numbers vary temporally during the healing process and may differ between acute and chronic wounds. Alteration of these parameters, which were not assessed, might additionally contribute to the androgenic response.

We have shown that manipulation of circulating androgen levels resulted in altered wound levels of a trio of pro-inflammatory cytokines with well established roles in wound healing. Castration (i.e. absence of DHT and testosterone) resulted in a reduction in wound IL-6 and TNF-α immunostaining and an increase in TGF-β1-positive cell numbers and protein. By contrast, treatment with MK-434 (i.e. absence of DHT alone) downregulated overall day-2-wound IL-6 levels but had little effect upon TNF-α and TGF-β1 levels suggesting that DHT is essential only for the regulation of IL-6 in this context, and that the modulation of TNF-α and TGF-β1 is less likely to contribute to the accelerated healing observed in MK-434-treated animals. That only a small proportion of wound inflammatory cells expressed 5α-reductase proteins suggests that they largely respond to DHT derived from systemic sources and synthesised locally from testosterone by epidermal keratinocytes. The specific contribution of IL-6 to a DHT-mediated delay in healing and increase in inflammation is intriguing in view of the known activation of the AR by IL-6 itself, in a MAPK-dependent fashion (Ueda et al., 2002). Depression of IL-6 (and potentially other pro-inflammatory cytokines) by MK-434 might contribute to the acceleration of wound repair by restraining an inflammatory response that retards healing progression.

Evaluation of cell-type-specific cytokine expression and secretion in response to in vitro androgen treatment highlighted a striking disparity between macrophages and fibroblasts. Whereas both testosterone and DHT provoked a significant increase in macrophage IL-6 gene expression without greatly affecting TNF-α or TGF-β1 mRNA levels, fibroblast IL-6 and TGF-β1 mRNA levels and protein secretion were significantly reduced by treatment with testosterone or DHT and TNF-α mRNA expression decreased in response to exposure to DHT. These findings are partially corroborated by previous studies which demonstrated that DHT and testosterone inhibit IL-6 production by oral and gingival fibroblasts and that DHT but not testosterone stimulates secretion of IL-6 by Kupffer cells (Coletta et al., 2002; Gornstein et al., 1999; Parkar et al., 1998; Schneider et al., 2003). The failure of testosterone to enhance macrophage TNF-α gene expression contrasts sharply with the situation in mice, in which testosterone has been shown to increase both basal and LPS-induced mRNA levels (Ashcroft and Mills, 2002). This disparity implies the existence of fundamental species differences and/or altered cell activation profiles. Overall, these data suggest that macrophages are more likely to be the candidate cellular effectors of androgen-stimulated inflammatory cytokine production during the early phases of wound healing than are fibroblasts, when this cell type constitutes a major population. Inhibition of cytokine release during the later repair phases, when fibroblasts predominate, may modulate tissue replacement and remodelling processes.

Intriguingly, both the MAPK- and the PI 3-kinase-pathways appear to be crucial for DHT-mediated induction of macrophage IL-6 gene expression and, moreover, the PI 3-kinase pathway also for the inhibition of pro-inflammatory cytokine gene expression by fibroblasts. Previous studies have shown that androgens, in non-genomic pathways, modulate PI 3-kinase signalling (Ashcroft et al., 1999b; Castoria et al., 2003; Baron et al., 2004). However, this is the first report of androgenic regulation of cytokine expression in fibroblasts utilising this signal transduction mechanism. That similar intermediates are involved in the up- or down-regulation of a specific gene depending upon the cell type might underlie the diverse effects of DHT in patho-physiological processes. In this regard, androgens play vital roles in the development and differentiation of several organs and in the maintenance of cellular homeostasis (George and Peterson, 1988; Sciote et al.,

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2001; Kapur and Reddi, 1989). In addition to the interactions with kinase pathways, androgenic responses are also influenced by TGF-β superfamily signalling intermediates in a reciprocal fashion. Cross-talk between the TGF-β- and androgen- and, indeed, MAPK-signalling pathways is mediated under specific circumstances in vitro by the TGF-β-activated transcription factor Smad3 and there is increasing evidence that direct interaction between Smad3 and AR can modulate each other's DNA-binding- and transcriptional-activation-activities (Hayes et al., 2001; Kang et al., 2001; Chipuk et al., 2001; Kamaraju and Roberts, 2005). Moreover, delayed activation of MAPK pathways depends on Smad signalling, and MAPK signalling modulates Smad pathways, such that Smad and AP-1 family members cooperate in transcriptional responses (Engel et al., 1999; Tardif et al., 2001). Although the significance of such regulatory associations in vivo is poorly characterised, they may have important implications for a range of androgen-linked pathological conditions including prostate cancer, atherosclerosis and renal disease (Adams et al., 1995; Baylis, 1994; Benson et al., 1985).

Smad3 appears to be a negative regulator of wound healing that retards closure and promotes inflammation (Ashcroft et al., 1999b). In a previous investigation, we utilised a cutaneous wound healing model in Smad3$^{-/-}$ mice to show that the modulation of in vivo wound repair by androgens directly or indirectly requires a functional Smad3 signalling pathway (Ashcroft et al., 2003a). The current study, employing the same murine model, has provided supplementary evidence that Smad signalling intermediates contribute to androgenic responses and documents a potential role for Smad3 in the regulation of pro-inflammatory cytokine production. The reduction in wound inflammatory and TNF-α-positive cell numbers and elevation of TGF-β1-positive cell numbers observed in castrated wild-type mice were not replicated in Smad3$^{-/-}$ animals. Moreover, our data suggest that androgens tightly regulate the expression of a number of Smad proteins both in vivo and in vitro. Castration of rats induced a significant increase in wound Smad7 immunostaining, paralleling a similar response observed in the ventral prostate of castrated animals (Brodin et al., 1999). Numbers of Smad4-expressing cells in the ventral prostate were reduced following castration and, here, we reveal that wound Smad4-positive cell numbers are reduced by MK-434 treatment and castration on day 6 post injury. Although wound phospho-Smad3 levels were unaffected by either MK-434 treatment or castration and would hence appear to be androgen-independent, it is possible that these androgen manipulations modulate other aspects of Smad3 function such as its nuclear shuttling or transcriptional activity.

The effects of in vitro androgen treatment on macrophage Smad expression have not previously been examined. We have shown that androgens stimulate expression of the Smad3 and Smad7 genes and that, in the case of Smad7, DHT is a significantly more potent inducer than testosterone. Overall, wound Smad7-immunopositive cell numbers were, however, elevated in castrated rats, a response for which cell types other than macrophages may be responsible. Also intriguing is the finding that wound endothelial cells (and those in wounds from castrated animals in particular) express especially high levels of Smad7 protein (compared with Smad3 and Smad4). In the light of a previous study, which demonstrated that overexpression of Smad7 abrogates the inhibitory effect of TGF-β2 on cultured endothelial cell proliferation (Funaki et al., 2003), this suggests that Smad7 acts to oppose the direct inhibition of angiogenesis by TGF-β in vivo. Furthermore, Smad7 inhibits TGF-β-activated Smad3 signalling, which implies that its in vivo upregulation of Smad7 by castration may act as a brake to limit the deleterious effects of Smad3 on wound healing, alone or in association with AR.

In summary, the data presented provide further evidence in support of the classification of androgens, in particular DHT, as inhibitors of wound repair (Fig. 6). Castrated rats heal wounds more rapidly and with reduced inflammation compared with controls. Furthermore, we have shown that blocking the conversion of testosterone to DHT accelerates healing and reduces wound IL-6 levels, suggesting that the negative effects of testosterone are through its metabolism to DHT. These findings may have important implications for male athletes who misuse DHT or other steroids that are metabolised to DHT. There is evidence to suggest that synthetic steroids whose actions are purely anabolic are functionally distinct from DHT in terms of their effects on wound repair (Demling and Orgill, 2000). The identification of elevated circulating DHT as a potential risk factor for venous ulceration might prove valuable in the targeting of 'at-risk' groups, and inhibition of 5α-reductases either topically or systemically might represent a new therapeutic strategy to accelerate healing in elderly males.

Materials and Methods

Wound healing experiments

Smad3$^{-/-}$ (Smad3$^{ex8/ex8}$) mice were generated by targeted disruption of the *SMAD3* gene by homologous recombination (Yang et al., 1999). Six-week-old male mice (wild-type, Smad3$^{-/-}$) were anaesthetised with methoxyfluorane and the dorsum shaved and cleaned with alcohol. A sub-group of mice had undergone castration (or sham procedure) two weeks prior to wounding. Two 1-cm full-thickness incisional wounds equidistant from the midline were made through the skin and panniculus carnosus muscle. Wounds were harvested at day 3 post-wounding (n=4-5 per treatment group) and were processed in formalin for wax embedding. Cardiac puncture was performed and were sera stored at –70°C for testosterone EIA measurements (ICN diagnostics, UK).

Eight-week-old male Sprague-Dawley rats were anaesthetised with isoflurane, and the dorsal surface was shaved and cleansed using ethanol. Four 1-cm dorsal incisions were made through the skin and underlying panniculus carnosus muscle, 5 cm and 10 cm caudal to the base of the skull and 1 cm either side of the midline. A subset of the rats had been castrated two weeks prior to wounding. Endogenous 5α-reductase activity was blocked in a further subgroup day by gavage of MK-434 (a gift from Merck, Hoddesdon, UK) 1 mg per day for 10 days prior to wounding and thereafter until the day of wound excision. Postoperatively, the animals were housed together according to treatment group (intact, MK-344-treated, castrated), and wounds were excised at days 2 (n=6-7 per treatment group) and day 6 (n=5-6) post-wounding. Each wound was bisected immediately after excision; one half was snap-frozen in liquid nitrogen and stored at –80°C and the other processed in formalin for wax embedding. Serum was collected from blood removed by cardiac puncture and peritoneal macrophages were isolated.

Histology, immunohistochemistry and image analysis

Histological sections (5 μm thickness) were prepared from wound tissue fixed in 10% buffered formalin and embedded in paraffin. Sections taken from the centre of each wound were stained with hematoxylin and eosin (H and E), or subjected to immunohistochemistry with antibodies raised against IL-6 (goat), TNF-α (goat) (both R&D Systems, Oxon, UK), AR (rabbit), Smad4 (goat), Smad7 (goat) 5α-R 1 (rabbit), 5α-R 2 (goat) (all Santa Cruz, Wembley, UK), Smad3 (rabbit; Cambridge BioScience, Cambridge, UK), ED1 (mouse; Serotec, Oxford, UK), TGF-β1 (rabbit; Promega, Southampton, UK) and His48 (mouse; BD Biosciences, Oxford, UK). As a negative control, sections on each slide were treated with PBS instead of the primary antibody; in all cases these showed no positive staining. Primary antibody was detected using the Vectastain ABC peroxidase kit as indicated (Vector Labs, Peterborough, UK). The images show regions from the centres of the wounds equidistant from the two lateral wound margins. Image analysis and quantification of wound areas and cell numbers per unit area (measured below the clot and above the panniculus muscle) were

performed with an Image-Pro Plus program (MediaCybernetics, Finchampstead, UK) as previously described (Ashcroft et al., 1997a).

Effects of androgens on macrophage activity

Rat peritoneal macrophages were isolated by intra-peritoneal lavage with sterile ice-cold sterile PBS and were pooled for subsequent studies. Cell viability was determined using Trypan Blue (Sigma). 2×10^6 cells/ml in suspension in serum-free Phenol-Red-free DMEM medium were treated for 6 hours with DHT (10^{-8} M) or testosterone (10^{-8} M), or left untreated. For the final 3 hours of the incubation period, one of the following inhibitors was added to the culture medium: 5 μM actinomycin D, 10 μM cycloheximide, 10 μM PD 98059 (MAPK inhibitor) or 100 nM wortmannin (PI 3-kinase inhibitor) (all Sigma). Additional macrophages were exposed for 18 hours to the following androgen concentrations: DHT (10^{-7} M or 10^{-8} M) or testosterone (10^{-7} M or 10^{-8} M).

Effects of androgens on fibroblast activity

Primary rat dermal fibroblasts were isolated by a previously described explant technique (Freshney, 1987). Cells were maintained in phenol red-free DMEM supplemented with 5% (v/v) heat-inactivated fetal calf serum. At passage five, cells were seeded to 6-well plates and at 90% confluence were switched to serum-free medium for a period of 24 hours. The fibroblasts were then treated for 6 hours with or without DHT or testosterone and for 3 hours with inhibitors as detailed above for macrophage culture.

Enzyme-linked immunosorbent assay (ELISA)

After the incubations described above the cells were pelleted by centrifugation and the supernatant was stored at –80°C for ELISA analysis of IL-6, TNF-α (both Biosource International, Nivelle, Belgium) and active TGF-β1 (Promega). The assay was performed according to manufacturers' instructions.

Real-time PCR

Total RNA was extracted from frozen wound tissue using Trizol (Invitrogen, Paisley, UK) and from macrophages and fibroblasts using RNeasy Mini kits (Qiagen, Crawley, UK) according to the manufacturer's instructions. cDNA was synthesised from 1 μg of wound or macrophage RNA using a reverse transcription kit (Promega) and separate AMV-reverse transcriptase (Roche, Lewes, UK). Quantitative Real-time PCR (qPCR) was performed using the SYBR Green I core kit (Eurogentec, Romsey, UK) following manufacturer's instructions and an Opticon qPCR thermal cycler (Genetic Research Instrumentation, Braintree, UK). For each primer set an optimal dilution was determined and melting curves used to determine the specificity of product amplification. Each sample was serially diluted over three orders of magnitude, and all samples were run on the same 96 well plate. PCR was carried out using primer pairs designed to murine TGF-β1, tumour necrosis factor (TNF)-α, IL-6, Smad3, Smad4, Smad7 and the housekeeping genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA (for normalisation). Primer sequences were: GAPDH (forward: TGCCACTCAGAA-GACTGTGG, reverse: GGATGCAGGGATGATGTTCT); 18S rRNA (forward: AGTCCCTGCCCTTTGTACACA; reverse: GATCCGAGGGCCTCACTAAAC); IL-6 (forward: TACCCCAACTTCCAATGCTC, reverse: TGGTCTTGGTCCTT-AGCCAC); TNF-α (forward: CTCTTCAAGGGACAAGGCTG, reverse: GGTA-TGAAGTGGCAAATCGG); TGF-β1 (forward: ATACGCCTGAGTGGCTGTCT, reverse: GTTTGGGACTGATCCCATTG); Smad3 (forward: AGGATTGCCAC-CAAAAATG, reverse: TTCTCTGTGATTGCCACTGC); Smad4 (forward: GGCATTGGTGTAGACGACCT, reverse: CGGTGGAGGTGAATCTCAAT); Smad7 (forward: CCAACTGCAGACTGTCCAGA, reverse: TTCTCCTCCCAG-TATGCCAC).

Immunoblotting

Total protein was extracted from rodent wound-tissue with detergent buffer. Extracted protein samples (n=6-7 per treatment group) were pooled and 1 mg was tested as described previously (Ashcroft et al., 1997b). Protein samples were separated by SDS-PAGE and then blotted onto 0.2 μm nitrocellulose membrane (Bio-Rad, Hemel Hempstead, UK) Membranes were blocked overnight. Sequential 1-hour incubations with primary antibodies and peroxidase-linked secondary antibodies were followed by detection using ECL Plus reagent (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions. Densitometric data were normalised to the signal for β-actin. Primary antibodies: anti-TNF-α, anti-TGF-β1, anti-IL-6 (as for immunohistochemistry) and anti-β-actin (mouse) (Sigma). Secondary antibodies: anti-rabbit (Amersham) and anti-goat (DakoCytomation, Ely, UK).

Statistical analysis

Statistical differences were determined by using Student's t-test, 1-way ANOVA or, for non-parametric data, Mann-Whitney U tests. A P value of <0.05 was considered significant.

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