Therapeutic antibodies for improved wound healing

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ABSTRACT

Therapeutic monoclonal antibodies (mAbs) are the fastest growing area of drug development, with an increasing number of diseases, including rheumatoid arthritis, multiple sclerosis and various forms of cancer, now amenable to treatment. Therapeutic mAbs bind to proteins or cells that are involved in the development of disease, impairing their ability to further contribute to the pathology. Currently, the treatment of acute and chronic wounds is an area of unmet clinical need. There are a number of proteins and cell types that are detrimental to wound healing and are up-regulated in the wound environment, especially in chronic wounds, with a reduction expected to improve healing outcomes. Therapeutic mAbs may therefore potentially provide a valuable new tool for wound treatment. This review explores the application of mAb therapies in wound healing.

Keywords: Wound healing, therapeutic antibodies.

INTRODUCTION

Wound healing is a process that is both dynamic and complex. The wound healing process involves the restoration of the cellular and tissue layers of the dermis and other soft tissue following an injury. Normal wound healing follows three distinct phases: inflammation, proliferation and remodelling. This healing process results in the replacement of regular skin structures with only a small amount of fibroblastic, mediated scar tissue. However, scars from acute wounds only retain approximately 80% tensile strength, when compared to healthy tissue[1]. Scarring can also be associated with functional impairment, such as reduced mobility, post-burn contractures and may be cosmetically unappealing[2].

In some cases there is a failure in the wound healing process, which leads to delayed healing, non-healing wounds, wound recurrence, keloid or hypertrophic scarring. Chronic wounds are a significant burden on health care systems and the community, costing the Australian health system over A$2.6 billion dollars per year[3]. Therefore, decreasing the scarring associated with acute wounds and improving the healing outcomes for chronic wounds is critical.

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Impaired healing involves alterations in the sensitive balance between the stimulation and inhibition of mediators during all stages of wound repair[4]. Most non-healing wounds are continually in a state of chronic inflammation[5]. This results in subsequent tissue responses that are aggravated by the hostile wound microenvironment[4]. The inflammatory response throughout impaired healing is caused by aberrations in the presence of various leukocyte subsets[6] and altered levels of cytokines (small cell signalling proteins) in the wound area[7]. These cytokines directly affect the remodelling process by mediating the action of proteolytic enzymes, including matrix metalloproteinases (MMPs)[8], and tissue inhibitors of MMPs (TIMPs)[9]. Myofibroblast cells, which typically are removed from the granulation tissue following wound closure, can also persist in chronic wounds; contributing to fibrosis and excessive scarring[8].

THERAPEUTIC MONOCLONAL ANTIBODIES (mAbs)

Therapeutic mAbs are mono-specific antibodies targeted to proteins that are elevated in various diseases (Figure 1). Binding to these mAbs impairs the proteins' contribution to the disease state which, in turn, leads to improved clinical outcomes. The greatest advantage of mAbs is their ability to bind with high specificity, providing direct targeting only to the site/s of pathology.

The most common method to develop mAbs involves immunising mice with the target protein (antigen) of interest (Figure 2)[10]. When mice develop a sufficient immune response, the animal is humanely killed, the spleen is harvested and the cells are isolated and grown in

culture. Importantly, each individual spleen cell (splenocyte) secretes a unique antibody, one that is different from those released from other splenocytes. A screening process is, therefore, required to identify splenocytes that secrete antibodies capable of binding strongly to the target protein. Once identified, these candidate cells are co-cultured with mouse myeloma cells under conditions that allow the cells to fuse together, with the resultant cells called hybridomas. This step is critical as it immortalises the antibody secreting cells (that is, the cells can theoretically be grown in culture forever), thereby providing an unlimited source of mAb which is purified from the cell culture medium.

Drawbacks of mAb therapies include the high cost due to the expense of drug manufacture (extensive purification is required to conform to good manufacturing practice) and the high dose required for efficacy in a number of diseases[11,12]. Other key factors for consideration when developing mAb therapies include: minimising mAb rapid degradation and clearance, identifying the optimal delivery route, maximising the drug's absorption and distribution, and minimising potential side effects. Recent research into the development of delivery systems has focused on using nanotechnological approaches to impart protection to the mAb *in vivo* and control release rates to overcome these issues[13].

There are currently more than 30 mAb therapies with clinical approval worldwide. Global sales are approaching US$40 billion per year, and hundreds of new mAb therapies are undergoing pre-clinical or clinical trials. Therapeutic mAbs are clinically approved to treat solid tumours, including colorectal carcinoma and squamous cell carcinoma of head/neck[14]. mAbs are available to treat haematological cancer, including chronic lymphocytic leukaemia[15]. Common inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, psoriasis and asthma, can also be treated with mAb therapies[16]. There are also mAb therapies available for other disorders including virus infection and wet age-related macular degeneration[17,18].

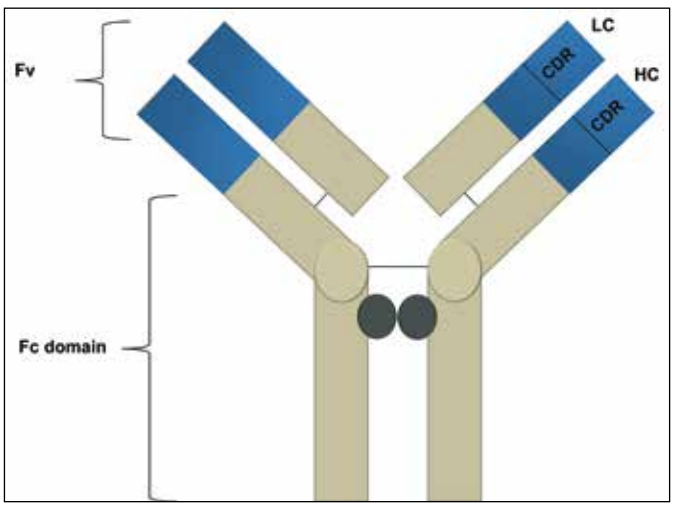

Figure 1: The structural elements of IgG, the most common mAb structure. IgGs are composed of two heavy chains (HC) two light chains (LC). The complementary determining region (CDR) of the variable fragment (Fv, blue) is responsible for binding to the target protein, which is either a protein involved in the development of pathology or a protein located on the surface of a target cell (i.e. cancer cell). The constant region (light brown) provides the antibody with structure and contains the Fc domain, which binds to the Fc receptor of immune cells, allowing the target cell to be killed. Adapted from Turner et al., 2015[13]

Therapeutic mAbs function by either neutralising the effect of the target protein, or by binding to cell surface proteins and triggering cell death (that is, cancer cells). Therapeutic mAbs neutralise the protein either by preventing binding to downstream targets or by masking the active site. For example, anti-Heat shock protein 90 (Hsp90) mAbs have been evaluated for the treatment of invasive candidiasis[19]. Hsp90 is a molecular chaperone that functions in the folding and stabilisation of proteins, but also facilitates a conformational change that is required for fungal viability[20]. *In vitro*, Hsp90 neutralisation

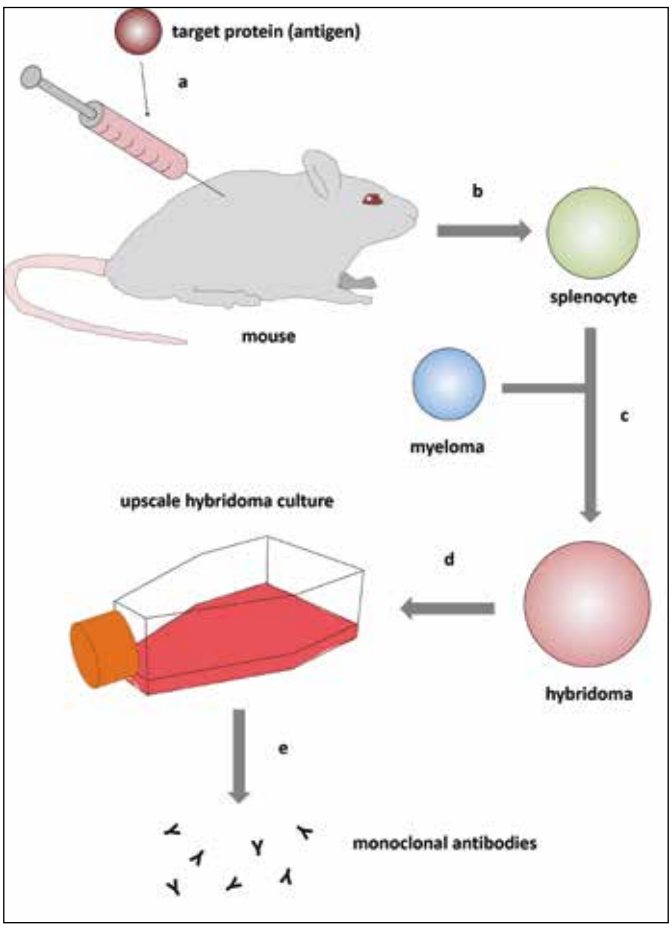

Figure 2: Production of mAbs. Mice are immunised against an antigen (the protein that the mAb will target) to stimulate the production of antibodies (a). The mouse is humanely killed, the spleen removed and individual splenocytes isolated (b). Each spleen cell secretes a unique antibody, one that no other cell can produce. These cells are co-cultured with myeloma cells under conditions that allow the cells to fuse together, resulting in the formation of a new cell type called a hybridoma (c). Following a screening process to identify the hybridoma that secretes the best performing mAb the hybridomas are cultured in large numbers to produce mAbs (d). The mAb can then be purified from the culture medium using chromatographic techniques (e)

has been reported to both increase anti-fungal activity and decrease resistance against antifungal agents[19]. Clinically approved mAbs that function by neutralisation include Abciximab (an inhibitor of platelet aggregation used to treat cardiovascular disease)[21], Ranibizumab (an inhibitor of blood vessel growth used to treat macular degeneration)[22] and Certolizumab pegol (an inhibitor of inflammation used to treat Crohn's disease)[23].

Effector functions are responsible for cell death (cytotoxicity). Three types of effector functions lead to cytotoxicity: complement activation, antibody-dependent cellular cytotoxicity (ADCC) and direct apoptosis (Figure 3)[24]. To induce complement activation, the mAb interacts with soluble blood protein components of the complement system[25]. This triggers the complement cascade and eventually leads to cell death[26]. ADCC is induced when the FcγRIII receptor located on the surface of predominantly B-cells (an important type of immune cell), including monocytes, macrophages, natural killer cells and neutrophils, is bound to the Fc region of the mAb[27], stimulating the release of cytotoxic molecules, including perforin and granzymes, which enter the target cell to trigger apoptosis. Antibody binding can also induce direct apoptosis through intracellular Ca^{2+} depletion, which in turn activates plasma membrane Ca^{2+} channels[28]. An influx of Ca^{2+} ions triggers intracellular apoptotic signalling pathways and cleaves caspase and poly (ADP-ri-bose) polymerase[29].

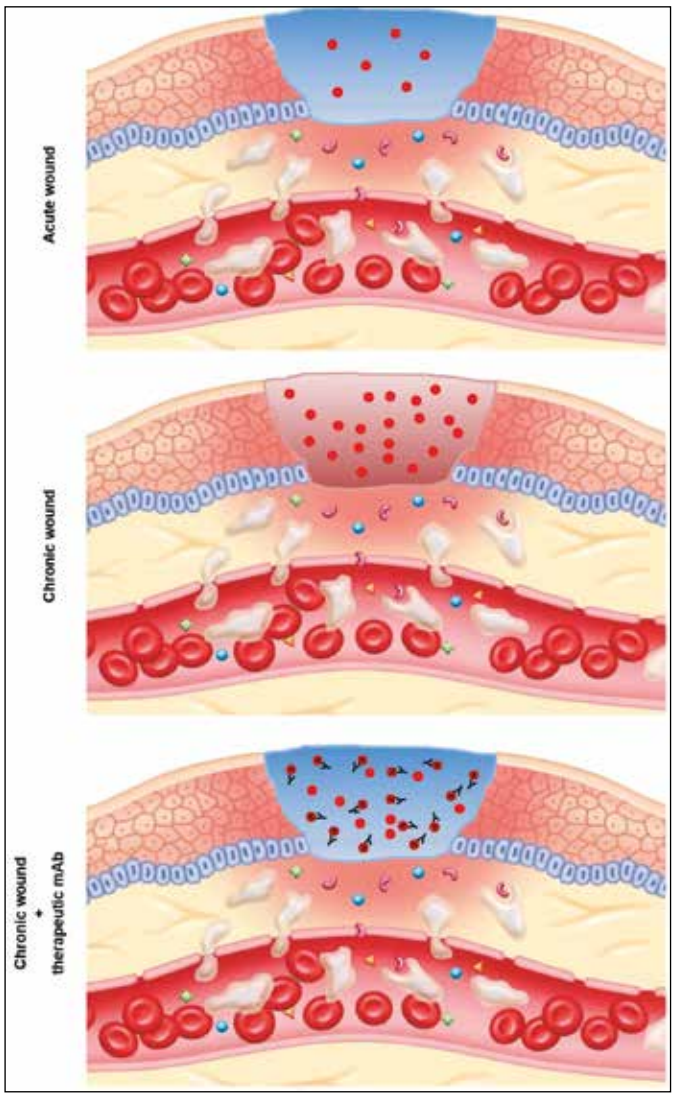

Figure 3: mAb neutralisation of proteins involved in chronic wound pathology. Schematic representation of a wound containing a protein (i.e. TGF-β, Flii or IL-6) that is up-regulated in chronic compared to acute wounds. This protein (red circle) can be neutralised (x) through the administration of therapeutic mAbs (Y), which bind to target protein and prevent their further involvement in the disease process, thereby leading to effective wound healing

Cell death can also be induced by conjugating the therapeutic mAb to cytotoxic payloads, including toxins and radioactive elements. Toxins conjugated to mAbs include maytansine, calicheamycin and auristatin[30]. Cytotoxic drugs and radioactive elements are typically used to treat cancer and target rapidly proliferating cells[31]. Toxic drugs disrupt different aspects of cell proliferation, including cell division and the repair, replication and translation of DNA[30]. Brentuximab vedotin, a mAb developed to treat haematologic malignancies, is conjugated to an anti-microtubule agent, monomethyl auristatin E (MMAE). The infused mAb binds to the surface of the target cancer cells (anti-CD30 protein), where Brentuximab vedotin is rapidly internalised and transported to lysosomes[32]. This leads to the release of MMAE, which then binds to tubulin, arresting the cell cycle and inducing programmed cell death.

mAbs provide a powerful therapeutic tool to interfere with proteins or cells involved in specific disease states. mAbs can be designed to target inflammatory cells and myofibroblasts, both up-regulated in chronic wounds, and proteins linked to delayed wound healing outcomes, including pro-inflammatory cytokines and MMPs. Hence, mAb therapy may soon prove to be a suitable treatment for the management of acute and chronic wounds.

POTENTIAL mAb TARGETS FOR WOUND TREATMENT

Transforming growth factor-β

The cytokine, transforming growth factor-β (TGF-β), is associated with multiple roles in wound healing. There are three isoforms of TGF-β (denoted as TGF-β_1, TGF-β_2 and TGF-β_3), which are 60–80% homologous. However, each form is responsible for different biological functions; mediating their effects through either Smad[33] or Smad-independent pathways[34].

TGF-β_1, the most common TGF-β in wounds[35], is up-regulated following injury and contributes to the recruitment of macrophages and fibroblasts into the wound area, the stimulation of collagen production, induction of angiogenesis, down regulation of proteinase activity, increased metalloproteinase inhibitor activity and induction of a myofibroblast phenotype[33]. TGF-β_1 inhibits the breakdown of the extracellular matrix (ECM)[36] and regulates ECM-cell interactions through integrin receptors[37]. Importantly, TGF-β_1 is also implicated in excessive scar formation and excessive disordered collagen deposition[38]. A number of fibrotic diseases, including scleroderma, glomerulonephritis, pulmonary fibrosis, liver cirrhosis, proliferative

vitreoretinopathy and postoperative peritoneal adhesions, are associated with an increase in TGF-β_1[39].

The neutralisation of TGF-β_1 (and also TGF-β_2 and TGF-β_3) with antibodies has been evaluated as a strategy to help reduce scar formation and fibrosis in a number of acute wound healing models. For example, dermal wounds in adult rats treated with anti-TGF-β antibodies prevented scar formation[40]. There was a reduction in macrophages, monocytes and blood vessels within the wounds of treated rats and a decrease in the deposition of both collagen and fibronectin. Anti-TGF-β antibody-treated mice also displayed the same tensile strength as control mice. Later studies confirmed that combined TGF-β_1/TGF-β_2 neutralisation could reduce scarring, with decreased inflammatory and angiogenic responses in a mouse dermal wound model, as well as reduced ECM deposition, without altering the tensile strength of the wound[39]. Improved wound healing in response to TGF-β antibody treatment has since been observed in various models including; rabbit eye wounding[41,42], rabbit flexor tendon wounds[43], mouse glaucoma surgery[42], mouse plastic surgery[44], rat nerve[45] and porcine skin wounds[46].

TGF-β neutralisation has also been evaluated in a chronic wound healing model (that is, hypertrophic scarring). Rabbit ear wounds treated with a generalised mAb that targeted all three TGF-β isoforms demonstrated a reduction in scar hypertrophy[38]. However, efficacy was only observed when antibody treatment was delayed until after epithelialisation was completed, indicating that TGF-β (at least at early time points) is a necessary component in the wound healing cascade.

Tumour necrosis factor-α

The pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α), mediates the activation, proliferation, or apoptotic death of cells[47]. TNF-α has been implicated in a number of inflammatory diseases, including chronic venous disease[48]. The level of TNF-α was significantly higher in wound fluid from biopsies of non-healing venous ulcers than healing ulcers[49]. Neutralisation of TNF-α was, therefore, predicted to alleviate the severity of inflammation in chronic wounds.

The anti-TNF-α mAbs, Infliximab, Adalimumab, Certolizumab pegol, Golimumab and Etanercept, have gained clinical approval for either rheumatoid arthritis or psoriasis treatment. The neutralisation of TNF-α induces the formation of regulatory macrophages with immunosuppressive properties[50], which then inhibit the proliferation of activated T-cells and trigger anti-inflammatory cytokine release[51].

Etanercept has been evaluated as a therapy for chronic wounds, and was found to neutralise TNF-α binding by up to 80%, reducing the cytotoxic effects of chronic wound fluid by approximately 30% on L929 fibroblasts[47]. A study of a rare ulcerative inflammatory cutaneous condition, refractory pyoderma gangrenosum ulcers, was performed where patients were treated with subcutaneous injections of Etanercept. There was an improvement in the time to heal and the size of the wound, whilst Etanercept caused no serious side effects and was well tolerated[52].

Infliximab has also been evaluated in some chronic wound healing trials, but the efficacy was less than observed for Etanercept[53], presumably caused by the development of anti-Infliximab antibodies[54]. To delay or even overcome the formation of anti-Infliximab antibodies, a periodic dose schedule or the simultaneous use of methotrexate and Infliximab has been investigated. However, these approaches only provided a marginal improvement[55]. mAbs containing less murine sequence than Infliximab (20% murine sequence), including Certolizumab pegol, have been developed with reduced antigenicity and has led to improved efficacy in rheumatoid arthritis patients[56].

Interleukin-6

Interleukin-6 (IL-6), a cytokine with multiple functions, is involved in the regulation of both immune responses, including B-cell and T-cell differentiation, and the acute inflammatory response[57]. IL-6 binds to the IL-6 receptor-a (IL-6R) on the cell surface, forming a complex that associates with the receptor subunit gp130 and leading to activation of various signalling pathways[58]. Through this mechanism, IL-6 is involved in a range of physiological processes, including epidermal proliferation, aging, cancer, bone metabolism, thrombopoiesis, neuronal cell differentiation and neuroprotection[59].

Aberrant IL-6 signalling is implicated in inflammatory diseases such as rheumatoid arthritis, Castleman's disease and osteoporosis[57]. Delayed wound healing is a hallmark of IL-6 knockout mice, caused by attenuated leukocyte infiltration and delays in re-epithelialisation, angiogenesis and collagen deposition[59]. IL-6 can also modulate α-smooth muscle actin, a marker of myofibroblasts[61]. By reducing inflammation, anti-IL-6 mAbs may provide a therapy for chronic wounds.

IL-6R neutralising mAb has been evaluated in alkali burns on mice cornea and resulted in a reduction in the vascularised area, a decreased infiltration of inflammatory cells and a significant inhibition of inflammatory-related molecule expression[62]. In other studies, localised administration of anti-IL-6R mAb to a collagen-induced cynomolgus monkey model of arthritis and in human patients after myocardial infarction both demonstrated a reduction in inflammation[63]. The anti-inflammatory effects of IL-6R neutralising mAbs may therefore provide a treatment option for chronic wounds.

Interleukin-1

The major cytokine, Interleukin-1 (IL-1), is involved in inflammation, pain and fever. There are three isoforms of IL-1 including IL-1 receptor antagonist and IL-1α, IL-1β, which are both agonists for IL-1 receptor binding[64]. IL-1β is activated through inflammasomes, innate immune complexes that sense intracellular danger[65], or through specific pathogen-associated molecular patterns[66]. Monocytes from the blood of patients with various auto-inflammatory diseases were found to release more IL-1β than monocytes from the blood of healthy individuals[67], and a reduction in IL-1β was predicted to reduce inflammation[64]. In clinical trials, IL-1β mAbs were demonstrated to normalise biochemical markers of inflammation and led to improved clinical outcomes[68]. Anti-IL-1β mAbs are now available for a range of auto-inflammatory diseases, including TNF receptor-associated periodic syndrome, cryopyrin-associated periodic syndrome and hyper-IgD syndrome[69]. As yet, no trials of IL-1 neutralisation have been performed for chronic wound treatment, but this therapeutic strategy may provide some clinical benefit.

Flightless I

Flightless I (Flii) is a highly conserved actin-remodelling protein and part of the gelsolin family[70]. Flii contains two domains; the leucine-rich repeat (LRR) domain, which mediates protein–protein interactions[71-73], and the actin-binding gelsolin-like domain. Flii is proposed to link the cytoskeletal network with specific signal transduction pathways[74].

Flii contributes to the regulation of cellular migration and proliferation[75], cell division[76], inflammatory cytokine production[77], toll-like receptor signalling[78], focal adhesion turnover[73] and transcriptional regulation[79]. Flii also plays a part in mediating cellular adhesion, hemidesmosome structure as well as collagen deposition[72,80]. In a series of salient experiments, impaired wound healing was observed in mice over-expressing Flii, whilst improved wound healing occurred in mice that had heterozygous Flii expression, when compared to controls[75]. This indicated that Flii negatively regulated wound healing.

The wound healing properties of a Flii neutralising antibody (FnAb) has been evaluated in acute wound models[74,75,81]. In murine incisional wounds, FnAb treatment demonstrated a significant reduction in wound size and an enhanced appearance, when compared to treatment with a non-specific control antibody[75]. Significant improvements in the healing of FnAb treated partial-thickness scald-burn injuries were also observed, when compared to control treatments[74].

FnAb has been evaluated in a porcine model of wound healing[82]. Porcine wounds, both incisional (5 cm) and excisional (6.25 cm^2), that were treated with FnAb at wounding and at 24 and 48 hours demonstrated a significant acceleration in re-epithelialisation and there was improve macroscopic appearance of early scars, as seen at day 35 post-wounding.

FnAb has also been evaluated as a therapeutic option for epidermolysis bullosa acquisita (EBA), a severe skin blistering disorder associated with structural skin and mucous membrane fragility[81]. EBA is caused by auto-immunity against type VII collagen, resulting in sub-epidermal blistering[83]. The compromised healing of these blisters can result in infections, scarring and even the development of squamous

cell carcinoma, often leading to metastasis and premature mortality[84]. Repeated application of FnAb during blister development in a murine model of EBA led to a significant decrease in the severity of skin blistering and improved the healing rate. Blisters that had already matured showed improved healing and a restoration of skin tensile strength, whilst early stage blisters demonstrated reduced severity upon treatment with FnAb.

DISCUSSION

Disruption of the pro-inflammatory cycle has been identified as a therapeutic strategy to heal chronic wounds[4]. Here, we present some evidence that mAbs targeting TGF-β, TNF-α, IL-6 and IL-1β may contribute to improved wound healing. Neutralisation impairs the down-stream effects of these cytokines; ultimately dampening inflammation and reducing both fibrosis and vascularisation.

Cytokines perform critical roles in acute wound repair, with tight regulation required to stop an inappropriate wound healing response[59]. Pro-inflammatory cytokines are up-regulated only transiently, before returning to basal levels. The neutralisation of these pro-inflammatory cytokines during this phase of wound healing may therefore have no effect or possibly impair the wound healing cascade. Anti-TGF-β treatment of hypertrophic scarring wounds in rabbits only demonstrated efficacy when treatment was delayed until after epithelialisation was completed[38]. Detrimental effects associated with pro-inflammatory cytokine neutralisation also include increasing the risk of infections[58] and malignancies[85]. Further studies are therefore required to optimise mAb treatment to improve safety and efficacy.

The neutralisation of other proteins up-regulated in the wound environment can also lead to improved wound healing, with the neutralisation of Flii significantly accelerating re-epithelialisation and improving short-term scar appearance. The persistent inflammatory response in chronic wounds is associated with elevated proteolytic activity, eventually overwhelming the normal tissue protective mechanisms[86,87]. In one study, protease activity has been reported to be 100-fold greater in chronic than acute wounds[88]. MMPs contribute to delayed healing by degrading growth factors[89], and adhesion proteins, including fibronectin and vitronectin[90], which prevent cell adhesion; a vital component of wound closure[3]. MMPs are therefore a possible target for mAb therapy. Serine proteinases, including cathepsin G, neutrophil elastase, and urokinase-type plasminogen activator, are also over-expressed in chronic wounds[87,90,91]. These wound proteases can specifically inactivate growth factors involved in wound repair, including vascular endothelial growth factor and platelet-derived growth factor[92-94]. The neutralisation of serine proteases may therefore reduce inflammation, providing a further target for mAb therapy.

The rapid degradation and clearance of therapeutic mAbs in hostile environments has led to an increase in the development of delivery systems. The key for these systems to improve clinical outcomes is based on the ability of these delivery systems to optimise the absorption and distribution whilst limiting the side effects[13]. Nanoparticle-based systems can give a controlled release profile and potentially protect the drug from degradation whilst shielding the patient from any immune responses associated with direct mAb infusion.

The aetiology of wounds varies widely, and prognosis is dependent on a number of biological factors. However, the relative abundance of various proteins, including cytokines, provides a useful indication as to the state of the wound. Measuring a cohort of key proteins, ones that are up-regulated in different wound types, has been identified as a strategy to individualise patient treatment[95,96], and would identify which therapeutic mAb would be appropriate for which wound. The development of assays that are both rapid and cheap may also be used to monitor the wound, indicating when mAb treatment/s should commence but also be concluded.

CONCLUSION

The mAb industry is the fastest growing pharmaceutical and is projected to account for 50% of all new drugs approved by 2014[97]. In concert with an increasing understanding of the biochemical basis of wound healing pathophysiology, more binding targets are likely to be identified. There is, therefore, anticipation that mAbs, combined with new delivery systems, will provide new therapeutic options for improved wound healing.

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