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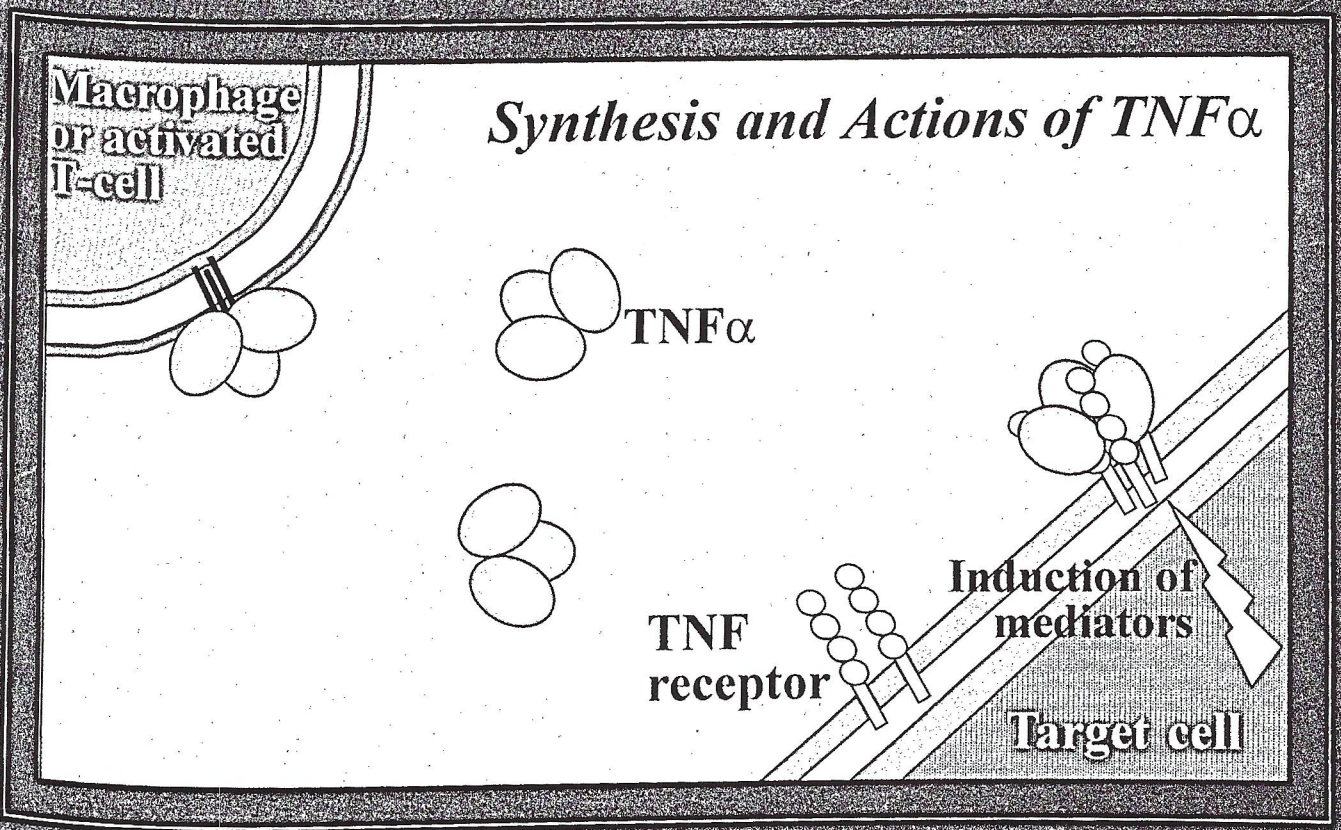
Annals of the Rheumatic Diseases

Advances in targeted therapies: TNFα blockade in clinical practice



Advances in targeted therapies: TNFα blockade in clinical practice

Editors: F C Breedveld, J R Kalden, J S Smolen

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Advances in targeted therapy: TNFα blockade in clinical p

Preface

Consensus statement

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Immunomodulation by thalidomide and thalidomide analogues

Laura G Corral, Gilla Kaplan

Tumour necrosis factor α (TNFα), a key cytokine involved in the host immune response, also contributes to the pathogenesis of both infectious and autoimmune diseases. To ameliorate the pathology resulting from TNFα in these clinical settings, strategies for the inhibition of this cytokine have been developed. Our previous work has shown that the drug thalidomide is a partial inhibitor of TNFα production in vivo. For example, when leprosy patients suffering from erythema nodosum leprosum (ENL) are treated with thalidomide, the increased serum TNFα concentrations characteristic of this syndrome are reduced, with a concomitant improvement in clinical symptoms. Similarly, we have found that in patients with tuberculosis, with or without HIV infection, short-term thalidomide treatment reduces plasma TNFα levels in association with an accelerated weight gain. In vitro, we have also shown that thalidomide partially inhibits TNFα produced by human peripheral blood mononuclear cells (PBMC) responding to stimulation with lipopolysaccharide (LPS). Recently, we found that thalidomide can also act as a costimulatory signal for T cell activation in vitro resulting in increased production of interleukin 2 (IL2) and interferon γ (IFNγ). We also observed a bidirectional effect on IL12 production: IL12 production is inhibited by thalidomide when PBMC are stimulated with LPS, however, IL12 production is increased in the presence of the drug when cells are stimulated via the T cell receptor. The latter effect is associated with upregulation of T cell CD40 ligand (CD40L) expression. Thus, in addition to its monocyte inhibitory activity, thalidomide exerts a costimulatory or adjuvant effect on T cell responses. This combination of effects may contribute to the immunomodulating properties of the drug.

To obtain drugs with increased anti-TNFα activity that have reduced or absent toxicities, novel TNFα inhibitors were designed using thalidomide as template. These thalidomide analogues were found to be up to 50 000 times more active than thalidomide. The compounds comprise two different types of TNFα inhibitors. One class of compounds, shown to be potent phosphodiesterase 4 (PDE4) inhibitors, are selective TNFα inhibitors in LPS stimulated PBMC and have either no effect or a suppressive effect on T cell activation. The other class of compounds also inhibit TNFα production, but do not inhibit PDE4 enzyme. These compounds are also potent inhibitors of several stimulate the anti-inflammatory cytokine IL10. Similarly to thalidomide, these drugs that do not inhibit PDE4 act as costimulators of T cells but are much more potent than the parent drug. The distinct immunomodulatory activity of these new TNFα inhibitors may potentially allow them to be used in the clinic for the treatment of a wide variety of immunopathological disorders of different aetiologies.

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TNFα is a key player in the immune response

TNFα is a pleiotropic cytokine produced primarily by monocytes and macrophages, but also by lymphocytes and NK cells. TNFα plays a central part in the host immune response to viral, parasitic, fungal and bacterial infections. The importance of TNFα and TNFα signalling through its receptors in the host immune response to disease has become clearer as a result of a number of seminal studies. For example, mice genetically deficient in TNFα have a significantly reduced humoral immune response to adenovirus infection.[1] In *Leishmania major* infection, TNFα signalling is important for protection as mice lacking TNFα p55 receptor (TNFR-p55) show delayed elimination of the parasites compared with controls and the lesions formed failed to resolve.[2] Mice deficient in TNFR-p55 are also significantly impaired in their ability to clear infection with *Candida albicans* and readily succumb to the infection. TNFα signalling is also crucial in resisting *Streptococcus pneumoniae* infections in mice.[3] In addition, TNFα is essential for protection against murine tuberculosis. TNFR-p55 deficient mice have been shown to be more susceptible to tuberculosis infection. When TNFα was neutralised in vivo by monoclonal antibodies impaired protection against mycobacterial infection was observed.[4,5] The data from both models also established that TNFα and the TNFR- p55 are essential for production of reactive nitrogen intermediates by macrophages early in infection.

TNFα contributes to disease pathogenesis

Although TNFα is crucial to the protective immune response, it also plays a part in the pathogenesis of both infectious and autoimmune diseases. Increased concentrations of TNFα have been shown to trigger the lethal effects of septic shock syndrome.[6] TNFα has also been implicated in the development of cachexia, the state of malnutrition that complicates the course of chronic infections and many cancers.[7] In rheumatoid arthritis, TNFα is a

Recently, it has been shown that treatment of patients with neutralising anti-TNFα antibodies produces a dramatic reduction in disease activity in this condition.[8] Similarly, it has been shown that in inflammatory bowel disease, neutralisation of TNFα results in a profound amelioration of clinical symptoms.[9 10] Reductions in TNFα levels have also been linked with a significant reduction of clinical symptoms in leprosy patients with ENL, including fever, malaise, and arthritic and neuritic pain.[11] In tuberculosis patients, reduction of TNFα levels was associated with accelerated weight gain.[12]

Thalidomide inhibits TNFα production by monocytes

The pathology associated with TNFα production is profound and in many diseases leads to significant morbidity and mortality. This has led to a concerted effort to discover drugs that will down regulate the production of this cytokine. Agents conventionally used in these diseases may inhibit TNFα production, but are also often broadly immunosuppressive (for example, cyclosporin A and corticosteroids) and therefore associated with extensive side effects.[13] Drugs that are potentially more specific in inhibiting TNFα are under active investigation and development. Our previous work has shown that the drug thalidomide (α-N-phthalimidiglutarimide) is a relatively selective inhibitor of TNFα production by human monocytes in vivo. This property of thalidomide was first described in leprosy patients with ENL, an acute inflammatory complication of lepromatous leprosy that is accompanied by increased serum TNFα levels. Thalidomide treatment of patients with ENL was shown to induce a prompt reduction of TNFα serum levels with a concomitant abrogation of clinical symptoms.[11] Furthermore, in patients with tuberculosis, with or without concomitant HIV infection, thalidomide treatment was found to both decrease plasma TNFα protein levels as well as monocyte TNFα mRNA levels. This decrease was associated with an accelerated weight gain.[12] In a rabbit model of mycobacterial meningitis, thalidomide treatment combined with antibiotics produced a marked reduction in TNFα levels, leucocytosis, and brain disease.[14] In addition, thalidomide inhibited TNFα serum levels in mice challenged with LPS thus partially protecting the animals from septic shock.[15]

In vitro, we have found that thalidomide selectively reduces the production of TNFα by human monocytes cultured in the presence of both LPS and mycobacterial products.[16] However, this inhibition was only partial (50% to 70%) possibly because of the instability of the drug in aqueous solutions.[17] The mechanism by which thalidomide reduces TNFα production is still unclear. The drug seems to inhibit TNFα production by human monocytes in vitro in association with enhanced degradation of TNFα mRNA.[18] It also inhibits the activa-

Thalidomide has T cell costimulatory properties

Recently, we reported that thalidomide also has a hitherto unappreciated immunomodulatory effect: the drug was shown to costimulate human T cells in vitro, synergising with stimulation via the T cell receptor complex to increase IL2 mediated T cell proliferation and T cell IFNγ production.[23] Optimal T cell activation requires two signals.[24] The first signal or signal 1 is delivered by clustering of the T cell antigen-receptor-CD3 complex through engagement of specific foreign peptides bound to MHC molecules on the surface of an antigen presenting cell (APC). Signal 1 can be mimicked by crosslinking the T cell receptor (TCR) complexes with anti-CD3 antibodies. Signal 2 (or costimulation) is antigen independent and may be provided by cytokines or by surface ligands on the APC that interact with their receptors on the T cell. Costimulatory signals are essential to induce maximal T cell proliferation and secretion of cytokines, including IL2, which ultimately drive T cell clonal expansion. As antigenic stimulation in the absence of costimulatory signals leads to T cell anergy or apoptosis, costimulation is critically important in the induction and regulation of cellular immunity.

Thalidomide appears to act as a costimulator to T cells that have received signal 1 via the TCR.[23] In our experiments in vitro, stimulation of purified T cells with anti-CD3 antibodies, in the absence of signal 2, induced only minimal T cell proliferation. However, the addition of thalidomide to this cell culture system resulted in a concentration dependent increase in proliferative responses.[23 25] The thalidomide mediated costimulation of T cell proliferation was accompanied by increases in IL2 and IFNγ production. It is noteworthy that in the absence of anti-CD3, there was no T cell proliferative response to thalidomide, indicating that the drug is not mitogenic in itself. It is also interesting to note that in these experiments, thalidomide did not inhibit TNFα production by purified T cells stimulated by anti-CD3 antibodies. This is in contrast with the effects of the drug on TNFα produced by monocytes. As already described above, thalidomide inhibits monocyte TNFα production. The costimulatory effect of thalidomide was greater on the CD8+ T cells than on the CD4+ T cell subset.[23]

In addition to its effects on T cell proliferation and T cell cytokine production, we observed that thalidomide induced the upregulation of CD40L expression on activated T cells.[25 26] CD40L/CD40 interaction occurs early in the sequence of signalling events between T cells and antigen presenting cells (APC). Signalling through CD40 has been shown to activate APC and to induce expression of costimulatory molecules such as B7, as well as stimulating production of IL12.[27 28] Thus, CD40 signalling results in a stimulatory

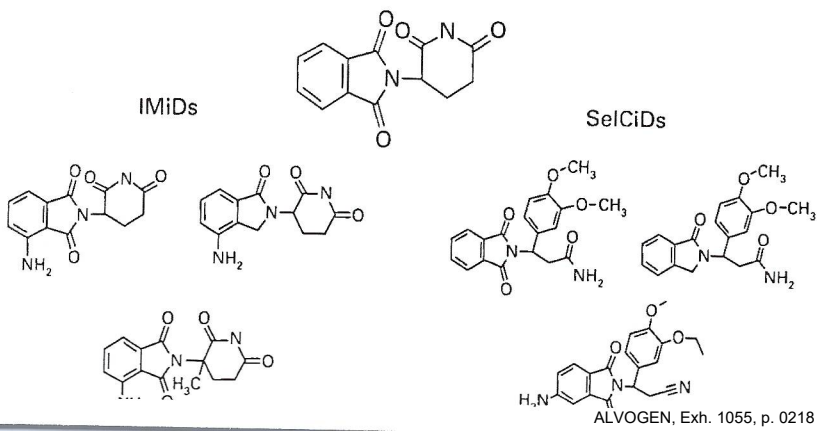
tial for the survival of CD8+ T cells and that in its absence these cells die or become anergic.[30]

These studies show that in addition to its inhibitory effect on the production of monocyte cytokines, thalidomide exerts a costimulatory or adjuvant effect on T cell responses. The immune modulating effects of the drug in patients may thus be attributable to a balance between the inhibition of production of monocyte cytokines, including TNFα, and the costimulation of T cell activity. The effects of thalidomide in vivo in HIV infected patients seem to reflect the costimulatory activity of the drug.[26] In a placebo controlled study to evaluate the effects of in vivo immunomodulation with thalidomide, the drug was administered for four weeks to HIV infected patients. Thalidomide treatment did not affect TNFα levels in these patients. In contrast, thalidomide treatment resulted in significant immune stimulation. This was reflected by increases in DTH responses and increased plasma levels of T cell activation markers such as soluble IL2 receptor (sIL2R) and soluble CD8 antigen. An earlier study of tuberculosis patients treated with thalidomide showed increased plasma levels of IFNγ suggesting an immunostimulatory effect of the drug.[12] Recently, patients suffering from sarcoidosis have shown consistent increases in sIL2R plasma levels after thalidomide treatment (Oliver *et al*, manuscript in preparation). In the same study, thalidomide treatment increased the proliferation of sarcoid patient T cells in response to concanavalin A in vitro. These results strongly suggest that thalidomide directly stimulates T cells in vivo in patients, corresponding to the T cell costimulatory properties of the drug observed in vitro in T cells from normal donors,[23 25] as well as in the T cells of HIV infected patients.[26]

Thalidomide analogues are improved TNFα inhibitors

In addition to being the drug of choice for the treatment of ENL, thalidomide has been shown to be useful in a number of clinical situations including rheumatoid arthritis, HIV associated aphthous ulcers and chronic graft versus host disease.[31-34] However, thalidomide is a potent teratogen and ingestion of the drug by a pregnant woman can lead to catastrophic birth defects.[35] In addition, thalidomide treatment is often accompanied by a number of side effects, including peripheral neuropathy.[36] Therefore, the use of thalidomide requires strict monitoring of all patients.[37] Thus, there is a pressing need to develop drugs with increased TNFα inhibitory activity and reduced or absent toxicities. Towards this end, structural analogues of thalidomide have been designed and synthesised at Celgene Corporation (Warren, New Jersey) and screened for inhibition of TNFα production. A large number of potent novel TNFα inhibitors were thus identified. Recently, some of these compounds were described.[20 38-40] On a molar basis, the more potent of these thalidomide analogues were found to be up to 50 000-fold more potent than thalidomide at inhibiting TNFα production by human PBMC stimulated by LPS in vitro. Furthermore, we have shown that some of these compounds retain high activity in LPS stimulated human whole blood.[40] In vivo, several of these new compounds showed improved activity in reducing LPS induced TNFα levels in mice[17] and in inhibiting the development of adjuvant arthritis in rats.[40a]

Thalidomide



Thalidomide analogues comprise two distinct classes of molecules

A group of thalidomide analogues, selected for their capacity to potently inhibit TNFα production by LPS stimulated PBMC, was further investigated (fig 1). When tested for their effect in vitro on LPS induced cytokines, different patterns of cytokine modulation were shown.[25] One class of compounds, class I or ImiDs (Immunomodulatory Imide Drugs) showed not only potent inhibition of TNFα but also marked inhibition of LPS induced monocyte IL1β and IL12 production. LPS induced IL6 was also inhibited by these drugs, albeit partially. These drugs were potent stimulators of LPS induced IL10, increasing IL10 levels by 200–300%. In contrast, the other class of compounds, class II or SelCiDs (Selective Cytokine Inhibitory Drugs), while still potently inhibiting TNFα production, had a more modest inhibitory effect on LPS induced IL1β and IL12, and did not inhibit IL6 even at high drug concentrations. In addition, SelCiDs produced a more modest IL10 stimulation (20–50% increases). In all of these characteristics, SelCiDs were more similar to thalidomide than ImiDs.[16 17]

Further characterisation of the SelCiDs showed that they are potent PDE4 inhibitors.[39] PDE4 is one of the major phosphodiesterase isoenzymes found in human myeloid and lymphoid lineage cells.[41] The enzyme plays a crucial part in regulating cellular activity by degrading the ubiquitous second messenger cAMP and maintaining it at low intracellular levels. Inhibition of PDE4 results in increased cAMP levels leading to the modulation of LPS induced cytokines including inhibition of TNFα.[42] Increasing intracellular cAMP levels have been shown to inhibit TNFα production

regulated. Interestingly, the IMiDs and thalidomide were found not to inhibit PDE4.[40]

In addition to the differential modulation of LPS induced monocyte cytokines, the two classes of compounds showed distinct effects on T cell activation. SelCiDs, the PDE4 inhibitors, had little effect on T cell activation causing only a slight inhibition of T cell proliferation. This effect was not unexpected as it is well established that increasing cAMP levels in T cells during the early phase of mitogen or antigen activation results in a decrease in proliferative potential.[41] On the other hand, IMiDs, the non-PDE4 inhibitors, were potent costimulators of T cells and increased cell proliferation dramatically in a dose dependent manner.[25] Similarly to thalidomide, these compounds had a greater costimulatory effect on the CD8+ T cell subset than on the CD4+ T cell subset (Corral *et al*, unpublished observation). IMiDs, when added to anti-CD3 stimulated T cells, also caused marked increases in the secretion of IL2 and IFNγ and induced the up-regulation of CD40L expression on T cells.[25] These findings show that in addition to their strong anti-inflammatory properties, IMiDs efficiently costimulate T cells with 100 to 1000 times the potency of the parent drug. The molecular target of these co-stimulatory cytokine modulating drugs is as yet unknown.

Thalidomide and IMiDs modulate cytokines differently according to cell type and stimulation pathway

As described above, thalidomide has been shown to inhibit IL12 production by LPS stimulated monocytes in vitro.[25 45] In vivo, however, thalidomide treatment of HIV infected[26] and *M tuberculosis* infected patients induced increases in plasma IL12 levels (Bekker *et al*, submitted data). Thalidomide treatment also resulted in increases in plasma IL12 levels in patients with scleroderma and sarcoidosis (Oliver *et al*, manuscripts in preparation). These dual and opposite effects of thalidomide may be explained by the differential modulation of cytokines according to target cell type and specific pathways of cellular stimulation.

IL12 is produced primarily by APC (monocytes/macrophages and dendritic cells) and is regulated by both T cell dependent and T cell independent pathways. LPS directly induces T cell independent IL12 production by APC, which is inhibited by thalidomide. In the T cell dependent pathway, on the other hand, the production of IL12 by the APC is induced primarily by the interaction of CD40 on the surface of the APC with CD40L on the surface of activated T cells.[28 46] When T cells were stimulated by anti-CD3, thalidomide and IMiDs treatment caused a significant stimulation of IL12 production.[25] Thalidomide and IMiDs also induced an up-regulation of CD40L on the surface of T cells.[25 26] Blockade of this pathway inhibits the production of IL12 and abolishes the stimulatory effect of thalidomide.[26] Interestingly, in HIV infected patients, the consistent increases in plasma IL12 levels induced by thalidomide treatment lagged behind the increases in T cell activation markers.[26] This observation suggested that IL12 production was augmented as a consequence of drug induced T cell activation.

The dichotomous nature of thalidomide cytokine modulation may explain the seemingly opposite effects observed in different clinical situations. When patients with Behçet's syndrome are treated with thalidomide, healing of inflammatory aphthous ulcers occurs, but is sometimes accompanied by exacerbation of erythema nodosum.[47] Similarly, the paradoxical worsening of graft versus host disease[48] and toxic epidermal necrolysis[49] reported in clinical trials of thalidomide may be a manifestation of the unsuspected immune stimulatory effect of this drug.

Potential clinical applications of thalidomide and thalidomide analogues

The thalidomide analogues discussed here seem to have retained different properties of the parent drug (table 1). The distinct immunomodulatory activities of these two classes of drugs suggest they may have applications in different immunopathological disorders. SelCiDs, which inhibit PDE4, may be used in clinical situations in which PDE4 inhibition and selective TNFα inhibition are beneficial. Therapeutic increase of intracellular cAMP levels by PDE4 inhibitors has anti-inflammatory effects, which may afford consequent benefits in a variety of diseases such as asthma,[50] atopic dermatitis[51] and rheumatoid arthritis.[52] Indeed, in an animal model of adjuvant arthritis, thalidomide derived PDE4 inhibitors have shown efficacy in suppressing the development of disease as measured by ankle swelling, hind limb radiographic changes and weight gain.[40a] The suppression of arthritis was accompanied by a reduction in TNFα and IL2 mRNA levels in the ankle joints of treated rats.

Table 1 Immunomodulatory profiles of thalidomide and thalidomide analogues

Thalidomide	*IMiDs*	*SelCIDs*
Inhibits LPS induced inflammatory cytokines TNFα and IL12	Strongly inhibit LPS induced inflammatory cytokines: TNFα, IL1β, IL6 and IL12	Strongly inhibit LPS induced inflammatory cytokines TNFα and IL12
Stimulates LPS induced anti-inflammatory cytokine IL10	Strongly stimulate LPS induced anti-inflammatory cytokine IL10	Stimulate LPS induced anti-inflammatory cytokine IL10
Costimulates T cell activation	Strongly costimulate T cell activation	Inhibit or have no effect on T cell

Other known selective PDE4 inhibitors, such as rolipram, have been reported to have dose limiting side effects, such as nausea and vomiting, which limit the therapeutic use of these drugs.[53 54] These side effects may be produced by the lack of specificity of these drugs—that is, the compounds inhibit one or more PDE isoenzymes in non-target tissues. For example, it is probable that the emetic activity of PDE4 inhibitors is attributable to an action of the drugs in the CNS.[55] Intensive effort is being directed towards identifying compounds with improved therapeutic ratios. Preliminary results with thalidomide derived PDE inhibitors indicate that these novel drugs are selective inhibitors of PDE4 and may be better tolerated than other PDE4 inhibitors, as they have not shown evidence of emesis in animals. One of these drugs has been recently shown to be well tolerated in a small human safety trial in the United Kingdom (D Stirling, personal communication).

The IMiDs, as thalidomide, are anti-inflammatory drugs that do not target PDE4. These compounds, in addition to their potential use to decrease inflammation, could also be useful in clinical settings where there is a defect in T cell function, as in HIV disease. HIV infection is accompanied by deficiencies in the production of IL12 and in the up-regulation of CD40L.[56 57] IL12 has been shown to restore HIV specific cell mediated immunity in vitro[58] and to increase HIV specific CTL responses in vitro[59] and in vivo.[60] Also, deficient IL12 responses in HIV infected patients can be restored in vitro by CD40L and IFNγ,[61] the same costimulatory factors induced by thalidomide and IMiDs. Thus, these drugs may eventually be used to restore or stimulate IL12 production in immune deficient patients.

IL12 has also been shown to exhibit potent anti-tumour activity in murine tumour models through various mechanisms including the stimulation of natural killer cell activity,[62] activation of CD8+ cytotoxic T cells[63] and increased IFNγ mediated anti-angiogenesis.[64] Thalidomide has also recently been reported to exhibit anti-tumour activity through the inhibition of angiogenesis in vivo.[65–68] However, this anti-angiogenic effect does not seem to be mediated by TNFα inhibition. Although these studies did not determine the mechanism of thalidomide's anti-angiogenic activity, it is conceivable that stimulation of IFNγ/IL12 levels may be at least partly responsible. One report indicates that thalidomide may have anti-angiogenic activity in multiple myeloma in humans.[69]

In summary, our recent findings that thalidomide and IMiDs preferentially costimulate CD8+ T cells and induce T cell dependent IL12 production suggest possible applications of these drugs in the control of viral infections[70 71] or in boosting anti-tumour immunity.[72 73] Also, there are anecdotal reports of the efficacy of thalidomide in treating refractory inflammatory bowel disease.[74–76] Recently, preliminary findings were announced from a pilot study with patients with Crohn's disease lando, FL). In this study, two third of the patients experienced a significant improvement in their condition. This therapeutic effect may be a combination of TNFα inhibition and CD8+ T cell stimulation.[77 78]

Conclusions

In several disease conditions such as septic shock, chronic infections and cancer, overproduction of TNFα is accompanied by severe toxicities. Thalidomide inhibits TNFα production in different diseases without causing the immunosuppression often associated with standard agents such as glucocorticoids and cyclosporin A. Our results indicate that the immunomodulating effects of thalidomide may occur via the inhibition of TNFα production and/or the stimulation of T cell responses, without the suppression of host immunity.

Recent efforts have concentrated on developing TNFα inhibitors that are efficient, safe and specific. The collaboration between Rockefeller University and Celgene Corporation scientists has led to the discovery of two different classes of immunomodulators derived from thalidomide and selected for their potent anti-TNFα inhibitory activity. Preliminary results indicate that at least some of these new compounds are non-toxic and non-teratogenic.[20] The two classes of thalidomide analogues, however, possess distinct properties. IMiDs are potent inhibitors of monocyte inflammatory cytokine production and also are strong costimulators of T cell activity. SelCiDs, on the other hand, are potent PDE4 inhibitors and thus, more selective inhibitors of TNFα. Unlike IMiDs, these compounds do not costimulate T cells but inhibit T cell activity. Thus, the two classes of compounds may prove to be useful in different clinical settings according to their immunomodulatory properties. The thalidomide analogues are being used as investigational tools in animal disease models to define mechanisms of pathogenesis and to continue to elucidate the mechanisms of drug action.

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EXHIBIT E2

Immunomodulation by thalidomide and thalidomide analogues

Laura G Corral, Gilla Kaplan

Tumour necrosis factor α (TNFα), a key cytokine involved in the host immune response, also contributes to the pathogenesis of both infectious and autoimmune diseases. To ameliorate the pathology resulting from TNFα in these clinical settings, strategies for the inhibition of this cytokine have been developed. Our previous work has shown that the drug thalidomide is a partial inhibitor of TNFα production in vivo. For example, when leprosy patients suffering from erythema nodosum leprosum (ENL) are treated with thalidomide, the increased serum TNFα concentrations characteristic of this syndrome are reduced, with a concomitant improvement in clinical symptoms. Similarly, we have found that in patients with tuberculosis, with or without HIV infection, short-term thalidomide treatment reduces plasma TNFα levels in association with an accelerated weight gain. In vitro, we have also shown that thalidomide partially inhibits TNFα produced by human peripheral blood mononuclear cells (PBMC) responding to stimulation with lipopolysaccharide (LPS). Recently, we found that thalidomide can also act as a costimulatory signal for T cell activation in vitro resulting in increased production of interleukin 2 (IL2) and interferon γ (IFNγ). We also observed a bidirectional effect on IL12 production: IL12 production is inhibited by thalidomide when PBMC are stimulated with LPS, however, IL12 production is increased in the presence of the drug when cells are stimulated via the T cell receptor. The latter effect is associated with upregulation of T cell CD40 ligand (CD40L) expression. Thus, in addition to its monocyte inhibitory activity, thalidomide exerts a costimulatory or adjuvant effect on T cell responses. This combination of effects may contribute to the immunomodulating properties of the drug.

To obtain drugs with increased anti-TNFα activity that have reduced or absent toxicities, novel TNFα inhibitors were designed using thalidomide as template. These thalidomide analogues were found to be up to 50 000 times more active than thalidomide. The compounds comprise two different types of TNFα inhibitors. One class of compounds, shown to be potent phosphodiesterase 4 (PDE4) inhibitors, are selective TNFα inhibitors in LPS stimulated PBMC and have either no effect or a suppressive effect on T cell activation. The other class of compounds also inhibit TNFα production, but do not inhibit PDE4 enzyme. These compounds are also potent inhibitors of several LPS induced monocyte inflammatory cytokines. Also, the latter compounds markedly stimulate the anti-inflammatory cytokine IL10. Similarly to thalidomide, these drugs that do not inhibit PDE4 act as costimulators of T cells but are much more potent than the parent drug. The distinct immunomodulatory activity of these new TNFα inhibitors may potentially allow them to be used in the clinic for the treatment of a wide variety of immunopathological disorders of different aetiologies.

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TNFα is a key player in the immune response

TNFα is a pleiotropic cytokine produced primarily by monocytes and macrophages, but also by lymphocytes and NK cells. TNFα plays a central part in the host immune response to viral, parasitic, fungal and bacterial infections. The importance of TNFα and TNFα signalling through its receptors in the host immune response to disease has become clearer as a result of a number of seminal studies. For example, mice genetically deficient in TNFα have a significantly reduced humoral immune response to adenovirus infection.[1] In *Leishmania major* infection, TNFα signalling is important for protection as mice lacking TNFα p55 receptor (TNFR-p55) show delayed elimination of the parasites compared with controls and the lesions formed failed to resolve.[2] Mice deficient in TNFR-p55 are also significantly impaired in their ability to clear infection with *Candida albicans* and readily succumb to the infection. TNFα signalling is also crucial in resisting *Streptococcus pneumoniae* infections in mice.[3] In addition, TNFα is essential for protection against murine tuberculosis. TNFR-p55 deficient mice have been shown to be more susceptible to tuberculosis infection. When TNFα was neutralised in vivo by monoclonal antibodies impaired protection against mycobacterial infection was observed.[4 5] The data from both models also established that TNFα and the TNFR- p55 are essential for production of reactive nitrogen intermediates by macrophages early in infection.

TNFα contributes to disease pathogenesis

Although TNFα is crucial to the protective immune response, it also plays a part in the pathogenesis of both infectious and autoimmune diseases. Increased concentrations of TNFα have been shown to trigger the lethal effects of septic shock syndrome.[6] TNFα has also been implicated in the development of cachexia, the state of malnutrition that complicates the course of chronic infections and many cancers.[7] In rheumatoid arthritis, TNFα is a critical mediator of joint inflammation and therefore an important therapeutic target.

Recently, it has been shown that treatment of patients with neutralising anti-TNFα antibodies produces a dramatic reduction in disease activity in this condition.[8] Similarly, it has been shown that in inflammatory bowel disease, neutralisation of TNFα results in a profound amelioration of clinical symptoms.[9 10] Reductions in TNFα levels have also been linked with a significant reduction of clinical symptoms in leprosy patients with ENL, including fever, malaise, and arthritic and neuritic pain.[11] In tuberculosis patients, reduction of TNFα levels was associated with accelerated weight gain.[12]

Thalidomide inhibits TNFα production by monocytes

The pathology associated with TNFα production is profound and in many diseases leads to significant morbidity and mortality. This has led to a concerted effort to discover drugs that will down regulate the production of this cytokine. Agents conventionally used in these diseases may inhibit TNFα production, but are also often broadly immunosuppressive (for example, cyclosporin A and corticosteroids) and therefore associated with extensive side effects.[13] Drugs that are potentially more specific in inhibiting TNFα are under active investigation and development. Our previous work has shown that the drug thalidomide (α-N-phthalimidiglutarimide) is a relatively selective inhibitor of TNFα production by human monocytes in vivo. This property of thalidomide was first described in leprosy patients with ENL, an acute inflammatory complication of lepromatous leprosy that is accompanied by increased serum TNFα levels. Thalidomide treatment of patients with ENL was shown to induce a prompt reduction of TNFα serum levels with a concomitant abrogation of clinical symptoms.[11] Furthermore, in patients with tuberculosis, with or without concomitant HIV infection, thalidomide treatment was found to both decrease plasma TNFα protein levels as well as monocyte TNFα mRNA levels. This decrease was associated with an accelerated weight gain.[12] In a rabbit model of mycobacterial meningitis, thalidomide treatment combined with antibiotics produced a marked reduction in TNFα levels, leucocytosis, and brain disease.[14] In addition, thalidomide inhibited TNFα serum levels in mice challenged with LPS thus partially protecting the animals from septic shock.[15]

In vitro, we have found that thalidomide selectively reduces the production of TNFα by human monocytes cultured in the presence of both LPS and mycobacterial products.[16] However, this inhibition was only partial (50% to 70%) possibly because of the instability of the drug in aqueous solutions.[17] The mechanism by which thalidomide reduces TNFα production is still unclear. The drug seems to inhibit TNFα production by human monocytes in vitro in association with enhanced degradation of TNFα mRNA.[18] It also inhibits the activation of the nuclear factor κB (NfκB),[19 20] a promoter for the transcription of TNFα as well as transcription of HIV-1.[21 22]

Thalidomide has T cell costimulatory properties

Recently, we reported that thalidomide also has a hitherto unappreciated immunomodulatory effect: the drug was shown to costimulate human T cells in vitro, synergising with stimulation via the T cell receptor complex to increase IL2 mediated T cell proliferation and T cell IFNγ production.[23] Optimal T cell activation requires two signals.[24] The first signal or signal 1 is delivered by clustering of the T cell antigen-receptor-CD3 complex through engagement of specific foreign peptides bound to MHC molecules on the surface of an antigen presenting cell (APC). Signal 1 can be mimicked by crosslinking the T cell receptor (TCR) complexes with anti-CD3 antibodies. Signal 2 (or costimulation) is antigen independent and may be provided by cytokines or by surface ligands on the APC that interact with their receptors on the T cell. Costimulatory signals are essential to induce maximal T cell proliferation and secretion of cytokines, including IL2, which ultimately drive T cell clonal expansion. As antigenic stimulation in the absence of costimulatory signals leads to T cell anergy or apoptosis, costimulation is critically important in the induction and regulation of cellular immunity.

Thalidomide appears to act as a costimulator to T cells that have received signal 1 via the TCR.[23] In our experiments in vitro, stimulation of purified T cells with anti-CD3 antibodies, in the absence of signal 2, induced only minimal T cell proliferation. However, the addition of thalidomide to this cell culture system resulted in a concentration dependent increase in proliferative responses.[23 25] The thalidomide mediated costimulation of T cell proliferation was accompanied by increases in IL2 and IFNγ production. It is noteworthy that in the absence of anti-CD3, there was no T cell proliferative response to thalidomide, indicating that the drug is not mitogenic in itself. It is also interesting to note that in these experiments, thalidomide did not inhibit TNFα production by purified T cells stimulated by anti-CD3 antibodies. This is in contrast with the effects of the drug on TNFα produced by monocytes. As already described above, thalidomide inhibits monocyte TNFα production. The costimulatory effect of thalidomide was greater on the CD8+ T cells than on the CD4+ T cell subset.[23]

In addition to its effects on T cell proliferation and T cell cytokine production, we observed that thalidomide induced the upregulation of CD40L expression on activated T cells.[25 26] CD40L/CD40 interaction occurs early in the sequence of signalling events between T cells and antigen presenting cells (APC). Signalling through CD40 has been shown to activate APC and to induce expression of costimulatory molecules such as B7, as well as stimulating production of IL12.[27 28] Thus, CD40 signalling results in a stimulatory feedback mechanism in which the activated APC amplifies the T cell response.[29] It has also been suggested that CD40L function is essen-

tial for the survival of CD8+ T cells and that in its absence these cells die or become anergic.[30]

These studies show that in addition to its inhibitory effect on the production of monocyte cytokines, thalidomide exerts a costimulatory or adjuvant effect on T cell responses. The immune modulating effects of the drug in patients may thus be attributable to a balance between the inhibition of production of monocyte cytokines, including TNFα, and the costimulation of T cell activity. The effects of thalidomide in vivo in HIV infected patients seem to reflect the costimulatory activity of the drug.[26] In a placebo controlled study to evaluate the effects of in vivo immunomodulation with thalidomide, the drug was administered for four weeks to HIV infected patients. Thalidomide treatment did not affect TNFα levels in these patients. In contrast, thalidomide treatment resulted in significant immune stimulation. This was reflected by increases in DTH responses and increased plasma levels of T cell activation markers such as soluble IL2 receptor (sIL2R) and soluble CD8 antigen. An earlier study of tuberculosis patients treated with thalidomide showed increased plasma levels of IFNγ suggesting an immunostimulatory effect of the drug.[12] Recently, patients suffering from sarcoidosis have shown consistent increases in sIL2R plasma levels after thalidomide treatment (Oliver *et al*, manuscript in preparation). In the same study, thalidomide treatment increased the proliferation of sarcoid patient T cells in response to concanavalin A in vitro. These results strongly suggest that thalidomide directly stimulates T cells in vivo in patients, corresponding to the T cell costimulatory properties of the drug observed in vitro in T cells from normal donors,[23 25] as well as in the T cells of HIV infected patients.[26]

Thalidomide analogues are improved TNFα inhibitors

In addition to being the drug of choice for the treatment of ENL, thalidomide has been shown to be useful in a number of clinical situations including rheumatoid arthritis, HIV associated aphthous ulcers and chronic graft versus host disease.[31–34] However, thalidomide is a potent teratogen and ingestion of the drug by a pregnant woman can lead to catastrophic birth defects.[35] In addition, thalidomide treatment is often accompanied by a number of side effects, including peripheral neuropathy.[36] Therefore, the use of thalidomide requires strict monitoring of all patients.[37] Thus, there is a pressing need to develop drugs with increased TNFα inhibitory activity and reduced or absent toxicities. Towards this end, structural analogues of thalidomide have been designed and synthesised at Celgene Corporation (Warren, New Jersey) and screened for inhibition of TNFα production. A large number of potent novel TNFα inhibitors were thus identified. Recently, some of these compounds were described.[20 38–40] On a molar basis, the more potent of these thalidomide analogues were found to be up to 50 000-fold more potent than thalidomide at inhibiting TNFα production by human PBMC stimulated by LPS in vitro. Furthermore, we have shown that some of these compounds retain high activity in LPS stimulated human whole blood.[40] In vivo, several of these new compounds showed improved activity in reducing LPS induced TNFα levels in mice[17] and in inhibiting the development of adjuvant arthritis in rats.[40a]

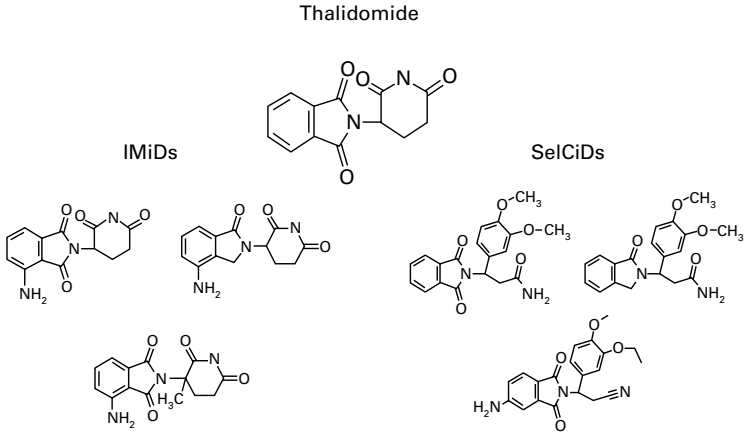

Figure 1 Chemical structures of thalidomide and selected thalidomide analogues.

Thalidomide analogues comprise two distinct classes of molecules

A group of thalidomide analogues, selected for their capacity to potently inhibit TNFα production by LPS stimulated PBMC, was further investigated (fig 1). When tested for their effect in vitro on LPS induced cytokines, different patterns of cytokine modulation were shown.[25] One class of compounds, class I or ImiDs (Immunomodulatory Imide Drugs) showed not only potent inhibition of TNFα but also marked inhibition of LPS induced monocyte IL1β and IL12 production. LPS induced IL6 was also inhibited by these drugs, albeit partially. These drugs were potent stimulators of LPS induced IL10, increasing IL10 levels by 200–300%. In contrast, the other class of compounds, class II or SelCiDs (Selective Cytokine Inhibitory Drugs), while still potently inhibiting TNFα production, had a more modest inhibitory effect on LPS induced IL1β and IL12, and did not inhibit IL6 even at high drug concentrations. In addition, SelCiDs produced a more modest IL10 stimulation (20–50% increases). In all of these characteristics, SelCiDs were more similar to thalidomide than ImiDs.[16 17]

Further characterisation of the SelCiDs showed that they are potent PDE4 inhibitors.[39] PDE4 is one of the major phosphodiesterase isoenzymes found in human myeloid and lymphoid lineage cells.[41] The enzyme plays a crucial part in regulating cellular activity by degrading the ubiquitous second messenger cAMP and maintaining it at low intracellular levels. Inhibition of PDE4 results in increased cAMP levels leading to the modulation of LPS induced cytokines including inhibition of TNFα.[42] Increasing intracellular cAMP levels have been shown to inhibit TNFα production in monocytes as well as in lymphocytes,[41 43] although it is not clear how this inhibition is

regulated. Interestingly, the IMiDs and thalidomide were found not to inhibit PDE4.[40]

In addition to the differential modulation of LPS induced monocyte cytokines, the two classes of compounds showed distinct effects on T cell activation. SelCiDs, the PDE4 inhibitors, had little effect on T cell activation causing only a slight inhibition of T cell proliferation. This effect was not unexpected as it is well established that increasing cAMP levels in T cells during the early phase of mitogen or antigen activation results in a decrease in proliferative potential.[44] On the other hand, IMiDs, the non-PDE4 inhibitors, were potent costimulators of T cells and increased cell proliferation dramatically in a dose dependent manner.[25] Similarly to thalidomide, these compounds had a greater costimulatory effect on the CD8+ T cell subset than on the CD4+ T cell subset (Corral *et al*, unpublished observation). IMiDs, when added to anti-CD3 stimulated T cells, also caused marked increases in the secretion of IL2 and IFNγ and induced the up-regulation of CD40L expression on T cells.[25] These findings show that in addition to their strong anti-inflammatory properties, IMiDs efficiently costimulate T cells with 100 to 1000 times the potency of the parent drug. The molecular target of these co-stimulatory cytokine modulating drugs is as yet unknown.

Thalidomide and IMiDs modulate cytokines differently according to cell type and stimulation pathway

As described above, thalidomide has been shown to inhibit IL12 production by LPS stimulated monocytes in vitro.[25 45] In vivo, however, thalidomide treatment of HIV infected[26] and *M tuberculosis* infected patients induced increases in plasma IL12 levels (Bekker *et al*, submitted data). Thalidomide treatment also resulted in increases in plasma IL12 levels in patients with scleroderma and sarcoidosis (Oliver *et al*, manuscripts in preparation). These dual and opposite effects of thalidomide may be explained by the differential modulation of cytokines according to target cell type and specific pathways of cellular stimulation.

IL12 is produced primarily by APC (monocytes/macrophages and dendritic cells) and is regulated by both T cell dependent and T cell independent pathways. LPS directly induces T cell independent IL12 production by APC, which is inhibited by thalidomide. In the T cell dependent pathway, on the other hand, the production of IL12 by the APC is induced primarily by the interaction of CD40 on the surface of the APC with CD40L on the surface of activated T cells.[28 46] When T cells were stimulated by anti-CD3, thalidomide and IMiDs treatment caused a significant stimulation of IL12 production.[25] Thalidomide and IMiDs also induced an up-regulation of CD40L on the surface of T cells.[25 26] Blockade of this pathway inhibits the production of IL12 and abolishes the stimulatory effect of thalidomide.[26] Interestingly, in HIV infected patients, the consistent increases in plasma IL12 levels induced by thalidomide treatment lagged behind the increases in T cell activation markers.[26] This observation suggested that IL12 production was augmented as a consequence of drug induced T cell activation.

The dichotomous nature of thalidomide cytokine modulation may explain the seemingly opposite effects observed in different clinical situations. When patients with Behçet's syndrome are treated with thalidomide, healing of inflammatory aphthous ulcers occurs, but is sometimes accompanied by exacerbation of erythema nodosum.[47] Similarly, the paradoxical worsening of graft versus host disease[48] and toxic epidermal necrolysis[49] reported in clinical trials of thalidomide may be a manifestation of the unsuspected immune stimulatory effect of this drug.

Potential clinical applications of thalidomide and thalidomide analogues

The thalidomide analogues discussed here seem to have retained different properties of the parent drug (table 1). The distinct immunomodulatory activities of these two classes of drugs suggest they may have applications in different immunopathological disorders. SelCiDs, which inhibit PDE4, may be used in clinical situations in which PDE4 inhibition and selective TNFα inhibition are beneficial. Therapeutic increase of intracellular cAMP levels by PDE4 inhibitors has anti-inflammatory effects, which may afford consequent benefits in a variety of diseases such as asthma,[50] atopic dermatitis[51] and rheumatoid arthritis.[52] Indeed, in an animal model of adjuvant arthritis, thalidomide derived PDE4 inhibitors have shown efficacy in suppressing the development of disease as measured by ankle swelling, hind limb radiographic changes and weight gain.[40a] The suppression of arthritis was accompanied by a reduction in TNFα and IL2 mRNA levels in the ankle joints of treated rats.

Table 1 Immunomodulatory profiles of thalidomide and thalidomide analogues

Thalidomide	*IMiDs*	*SelCIDs*
Inhibits LPS induced inflammatory cytokines TNFα and IL12	Strongly inhibit LPS induced inflammatory cytokines: TNFα, IL1β, IL6 and IL12	Strongly inhibit LPS induced inflammatory cytokines TNFα and IL12
Stimulates LPS induced anti-inflammatory cytokine IL10	Strongly stimulate LPS induced anti-inflammatory cytokine IL10	Stimulate LPS induced anti-inflammatory cytokine IL10
Costimulates T cell activation	Strongly costimulate T cell activation	Inhibit or have no effect on T cell activation
Does not inhibit PDE4	Do not inhibit PDE4	Strongly inhibit PDE4

Other known selective PDE4 inhibitors, such as rolipram, have been reported to have dose limiting side effects, such as nausea and vomiting, which limit the therapeutic use of these drugs.[53 54] These side effects may be produced by the lack of specificity of these drugs—that is, the compounds inhibit one or more PDE isoenzymes in non-target tissues. For example, it is probable that the emetic activity of PDE4 inhibitors is attributable to an action of the drugs in the CNS.[55] Intensive effort is being directed towards identifying compounds with improved therapeutic ratios. Preliminary results with thalidomide derived PDE inhibitors indicate that these novel drugs are selective inhibitors of PDE4 and may be better tolerated than other PDE4 inhibitors, as they have not shown evidence of emesis in animals. One of these drugs has been recently shown to be well tolerated in a small human safety trial in the United Kingdom (D Stirling, personal communication).

The IMiDs, as thalidomide, are anti-inflammatory drugs that do not target PDE4. These compounds, in addition to their potential use to decrease inflammation, could also be useful in clinical settings where there is a defect in T cell function, as in HIV disease. HIV infection is accompanied by deficiencies in the production of IL12 and in the up-regulation of CD40L.[56 57] IL12 has been shown to restore HIV specific cell mediated immunity in vitro[58] and to increase HIV specific CTL responses in vitro[59] and in vivo.[60] Also, deficient IL12 responses in HIV infected patients can be restored in vitro by CD40L and IFNγ,[61] the same costimulatory factors induced by thalidomide and IMiDs. Thus, these drugs may eventually be used to restore or stimulate IL12 production in immune deficient patients.

IL12 has also been shown to exhibit potent anti-tumour activity in murine tumour models through various mechanisms including the stimulation of natural killer cell activity,[62] activation of CD8+ cytotoxic T cells[63] and increased IFNγ mediated anti-angiogenesis.[64] Thalidomide has also recently been reported to exhibit anti-tumour activity through the inhibition of angiogenesis in vivo.[65–68] However, this anti-angiogenic effect does not seem to be mediated by TNFα inhibition. Although these studies did not determine the mechanism of thalidomide's anti-angiogenic activity, it is conceivable that stimulation of IFNγ/IL12 levels may be at least partly responsible. One report indicates that thalidomide may have anti-angiogenic activity in multiple myeloma in humans.[69]

In summary, our recent findings that thalidomide and IMiDs preferentially costimulate CD8+ T cells and induce T cell dependent IL12 production suggest possible applications of these drugs in the control of viral infections[70 71] or in boosting anti-tumour immunity.[72 73] Also, there are anecdotal reports of the efficacy of thalidomide in treating refractory inflammatory bowel disease.[74–76] Recently, preliminary findings were announced from a pilot study with patients with Crohn's disease refractory to standard treatments (Annual Digestive Disease Meeting, May 1999, Orlando, FL). In this study, two third of the patients experienced a significant improvement in their condition. This therapeutic effect may be a combination of TNFα inhibition and CD8+ T cell stimulation.[77 78]

Conclusions

In several disease conditions such as septic shock, chronic infections and cancer, overproduction of TNFα is accompanied by severe toxicities. Thalidomide inhibits TNFα production in different diseases without causing the immunosuppression often associated with standard agents such as glucocorticoids and cyclosporin A. Our results indicate that the immunomodulating effects of thalidomide may occur via the inhibition of TNFα production and/or the stimulation of T cell responses, without the suppression of host immunity.

Recent efforts have concentrated on developing TNFα inhibitors that are efficient, safe and specific. The collaboration between Rockefeller University and Celgene Corporation scientists has led to the discovery of two different classes of immunomodulators derived from thalidomide and selected for their potent anti-TNFα inhibitory activity. Preliminary results indicate that at least some of these new compounds are non-toxic and non-teratogenic.[20] The two classes of thalidomide analogues, however, possess distinct properties. IMiDs are potent inhibitors of monocyte inflammatory cytokine production and also are strong costimulators of T cell activity. SelCiDs, on the other hand, are potent PDE4 inhibitors and thus, more selective inhibitors of TNFα. Unlike IMiDs, these compounds do not costimulate T cells but inhibit T cell activity. Thus, the two classes of compounds may prove to be useful in different clinical settings according to their immunomodulatory properties. The thalidomide analogues are being used as investigational tools in animal disease models to define mechanisms of pathogenesis and to continue to elucidate the mechanisms of drug action.

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EXHIBIT E5

The role of angiogenesis in rheumatoid arthritis: recent developments

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Rheumatoid arthritis (RA) is characterised by synovial tissue leucocyte ingress and angiogenesis, or new blood vessel growth.[1–8] The disease is thought to occur as an immunological response to an as yet unidentified antigen. Even in early RA, some of the earliest histological observations are blood vessels.[9] A mononuclear infiltrate characterises the synovial tissue along with a luxuriant vasculature. Angiogenesis is integral to formation of the inflammatory pannus and without angiogenesis, leucocyte ingress could not occur.

Angiogenesis is regulated by a complex set of inducers and inhibitors. In this paper we will present representative examples of both angiogenesis inducers and inhibitors that may regulate RA neovascularisation (fig 1). In inflammatory states like RA, angiogenesis inducers outweigh angiogenesis inhibitors.

Angiogenesis inducers

ENDOGLIN AS AN ANGIOGENIC MEDIATOR

There are a number of angiogenesis inducers that may play a part in RA. Among these are endoglin, an endothelial glycoprotein, which contains an arginine-glycine-aspartic acid (RGD) motif, and also acts as an adhesion molecule.[10 11] Endoglin is a receptor for transforming growth factor β. Mice lacking the endoglin gene die from defective vascular development.[12] We have shown that endoglin is upregulated in RA synovial endothelial cells compared with normal synovial tissue endothelial cells.[13]

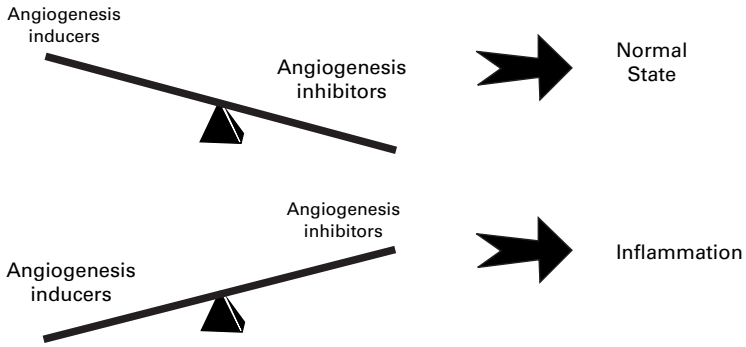

Figure 1 Balance of angiogenesis inducers and inhibitors in inflammatory states such as rheumatoid arthritis.

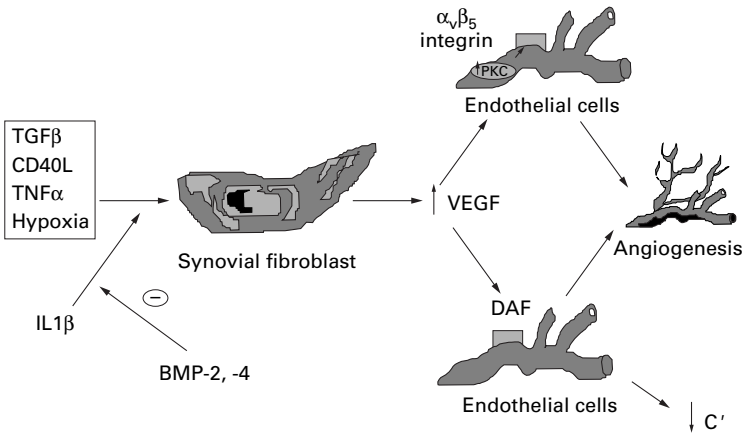

Figure 2 Proposed mechanism of action of cytokines inducing angiogenesis via integrin usage. Abbreviations are: decay accelerating factor (DAF), protein kinase C (PKC), tumour necrosis factor α, (TNFα), transforming growth factor β (TGFβ), and bone morphogenetic protein (BMP).

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

An angiogenic mediator that has attracted much attention recently is VEGF, which is an endothelial selective growth factor.[14] VEGF induces vascular permeability as well.[15] In human RA, several groups have described VEGF in the joints and serum of RA patients.[16–23] VEGF is inducible by hypoxia, which may occur in the inflamed joint.[17 24] Hypoxia inducible factor-1 (HIF-1), which is made of HIF-1α, and hydroxycarbon nuclear translocator (ARNT), contols many transcriptional responses to hypoxia by binding to hypoxia response elements of target genes like the VEGF gene.[25] In RA patients treated with anti-tumour necrosis factor α (TNFα), vascular deactivation occurs so that serum levels of VEGF fall along with clinical improvement.[26] A number of groups presented at the 1999 National American College of Rheumatology (ACR) meeting on the regulation of VEGF production by synovial fibroblasts, mainly from patients with RA (fig 2).[27–30] Not only do cytokines like interleukin 1 (IL1) and TNFα induce fibroblast expression of VEGF, but so does engagement of CD 40 ligand. Bone morphogenetic proteins (or BMPs), which induce formation of cartilage and bone, and seem to downregulate IL1 induced VEGF production. VEGF induces endothelial decay accelerating factor (DAF), which is cytoprotective against activated complement and may regulate endothelial proliferation and angiogenesis.

Similarly, the role of VEGF has been examined in animal arthritis models. In collagen induced in rats, a reduction in arthritis disease severity by angiogenesis inhibitors results in reduced serum levels of VEGF.[31] Administration of the fungal derivative TNP-470 in rodent arthritis leads to attenuated arthritis and serum VEGF production.[32] Administration of the VEGF receptor soluble flt-1 in mouse collagen induced arthritis results in attenuated arthritis.[33] These results indicate that modulation of angiogenesis may alter arthritis, at least in animal models. Hence, VEGF is probably an important mediator of angiogenesis in the RA joint.

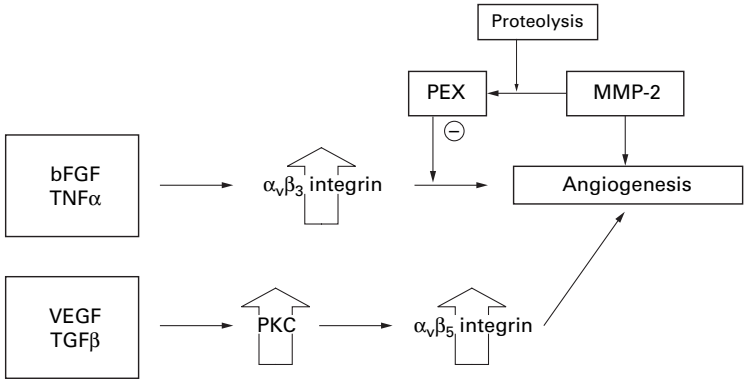

Figure 3 Proposed regulation of vascular endothelial growth factor (VEGF) in the RA joint. Abbreviations are protein kinase C (PKC) and matrix metalloproteinase-2 (MMP-2).

THE MECHANISM OF ACTION OF SOME ANGIOGENIC CYTOKINES: VIA ADHESION MOLECULES LIKE INTEGRINS

The invasion, migration, and proliferation of endothelial cells is regulated at least in part by the integrin family of cell adhesion molecules. Mice made deficient in αv integrins predominantly die in utero.[34] However, 20% of animals survive until term and die hours after birth of extensive brain and intestinal vascular abnormalities and haemorrhaging. It is very interesting that αvβ3 is minimally, if at all expressed on resting or normal blood vessels but is highly expressed in RA synovial blood vessels.[35] Some angiogenic factors like basic fibroblast growth factor (bFGF) and TNFα may act via integrins.[36] TNFα seems to act to mediate angiogenesis via αvβ3 integrin (fig 3). Angiogenesis can be inhibited by using αvβ3 antagonists that promote unscheduled programmed cell death (apoptosis) of newly sprouting blood vessels.[37] VEGF or transforming growth factor α appear to act via an alpha v beta 5 integrin mechanism using protein kinase C (PKC). This may turn out not to be a main mode of VEGF's action in RA as alpha v beta 5 integrin has been reported to be expressed in normal and osteoarthritis (OA) synovial tissue, but not RA synovial tissue.[38] None the less, the mechanisms by which some of these cytokines act to promote angiogenesis are rapidly becoming identified.

αvβ3 has been targeted using animal arthritis models. Administration of an αvβ3 antagonist ameliorates angiogenesis and decreases arthritis in a synovitis model in rabbits.[39] At the ACR meeting, there were several groups who have studied the role of this molecule in angiogenesis. An oral non-peptide αvβ3 antagonist ameliorates rat adjuvant induced arthritis, both prophylactically and therapeutically.[40] A proapoptotic αvβ3 antagonist composed of an RGD peptide linked to a heptapeptide dimer is therapeutic in mouse collagen induced arthritis.[41] However, this antagonist selectively homes to mouse arthritic versus normal joint endothelium and versus control organs. In this study, targeted apoptosis of synovial neovasculature resulted in improvement of arthritis. These studies indicate that this integrin can be modulated in vivo with resultant improvement in arthritis, possibly via effects on angiogenesis.

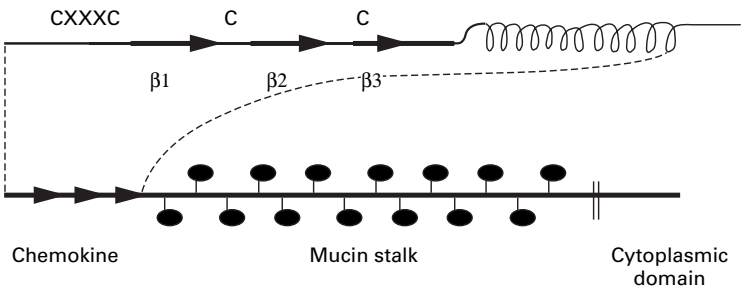

Figure 4 Structure of fractalkine, a CX_3C chemokine. (Adapted from Rollins BJ. Blood 1997;90:909.)

CHEMOKINES AS ANGIOGENIC MEDIATORS

Among other important mediators of angiogenesis are chemokines. Most chemokines are low molecular weight (8 kDa to 10 kDa) proteins that are predominantly known for their ability to recruit leucocytes.[42] Chemokines are divided into the C-X-C, CC, and $C\text{-}X_3\text{-}C$ families based on the presence or absence of an amino acid, X, between a pair of cysteine residues near the amino terminus of the protein. In collaboration with Dr Robert Strieter, Peter Polverini, and Steve Kunkel, our group found that monocyte/macrophage derived interleukin 8 (IL8), a prototype of the C-X-C chemokine subfamily, was angiogenic.[43] This factor seemed important in that synovial tissue macrophage derived chemotactic activity for endothelial cells in vitro and angiogenesis in vivo was significantly decreased if IL8 was immunodepleted. In general, chemokines like IL8, of the C-X-C class containing the amino acid E-L-R motif are angiogenic, while those lacking this motif are angiostatic.[44] Exceptions to this generalisation exist in that the C-X-C chemokine stem cell derived factor-1, which lacks the E-L-R motif is angiogenic.[45]

We have recently shown that fractalkine is the first chemokine described of the CX_3C class to mediate angiogenesis.[46 47] Fractalkine is termed for its fractal geometry and is the sole member of the CX_3C class of chemokines.[48] It contains a chemokine motif atop a mucin-like stick, the so called chemokine on a stick (fig 4). Factalkine is a unique chemokine in that it can also act as an adhesion molecule when cell bound. Fractalkine induces endothelial tube formation on the matrix Matrigel in vitro. Similarly fractalkine induces angiogenesis in Matrigel plugs implanted in mice in vivo. When fractalkine is immuodepleted from RA synovial fluids, the ability of these synovial fluids to chemoattract endothelial cells, a facet of the angiogenic response in vitro, is decreased. Hence, chemokines are probable contributors to RA angiogenic activity.

SOLUBLE ADHESION MOLECULES AS ANGIOGENIC MEDIATORS

Endothelial cells express soluble adhesion molecules, particularly upon cytokine stimulation. Cellular adhesion molecules can be shed from the cell surface and secreted. The function of these soluble adhesion molecules is unclear. A prevailing paradigm was that these molecules might serve an anti-inflammatory role by binding leucocytes, thus preventing

them from adhering to endothelium and entering inflamed tissues. We proposed the converse paradigm that these molecules may be proinflammatory in some cases. Our laboratory found that the cellular adhesion molecules sE-selectin and soluble vascular cell adhesion molecule-1 (sVCAM-1) are angiogenic.[49 50] It is probable that activated cells in the synovial milieu, such as endothelial cells, bear E-selectin and VCAM-1, which they then shed into the synovial fluid. These soluble adhesion molecules then interact with vascular endothelial cells via sialyl Le^x in the case of sE-selectin, and VLA-4, in the case of sVCAM-1 to mediate angiogenesis.[49 50] Additionally, the soluble adhesion molecules may bind other, as yet unidentified, receptors on endothelial cells that mediate angiogenesis. Interestingly, RA patients treated with monoclonal anti-TNFα have an element of endothelial deactivation in which treatment results in reduced levels of soluble E-selectin. Hence, these findings may be important clinically in RA.

GLYCOCONJUGATES AS ANGIOGENIC MEDIATORS

We have described another novel related angiogenic mediator in our laboratory. This antigen, termed 4A11, is an endothelial selective, cytokine inducible, endothelial angiogenic antigen.[51 52] We first raised monoclonal antibody (mAb) 4A11 by immunising mice with adherent cells from human rheumatoid synovial tissue. The mAb we produced recognised endothelium in the synovium, thymus, skin, and lymph node selectively, perhaps suggesting a role in cell homing to these regions. Moreover, the mAb was endothelial selective, recognising endothelium and keratinocytes only. This antigen is upregulated in RA compared with normal synovial tissue and is rapidly cytokine inducible in vitro, being stored in cytoplasmic vessicles and upregulated on the cell surface within 5 to 20 minutes of contact with cytokines. We have obtained a partial structure of the antigen recognised by mAb 4A11. The mAb detects $Lewis^y$-6 and H-5–2 antigens (Le^y/H). These structures are mainly recognised for their function as blood group antigens. Interestingly, these antigens are structurally related to the E-selectin ligand sialyl $Lewis^x$. Because of the angiogenic properties of soluble E-selectin, we hypothesised these endothelial antigens were released by activated endothelium and induced angiogenesis. Glucose analogues of these molecules or the glycolipids themselves induced a potent endothelial chemotactic response. Moreover, the glucose analogues are angiogenic in vivo in the corneal bioassays. mAb 4A11 abrogated the angiogenic responses. We reasoned that if these molecules mediated angiogenesis, they could be detected in clinical samples from RA patients. We found that soluble 4A11 antigen is increased in RA compared with OA serum and synovial fluid. These results describe a novel endothelium selective antigen that functions as an angiogenic mediator. As with the E-selectin and VCAM-1, it is probable that endothelial cells exposed to cytokines bear the 4A11 antigen, which is shed in the inflamed joint and mediates angiogenesis (fig 5).

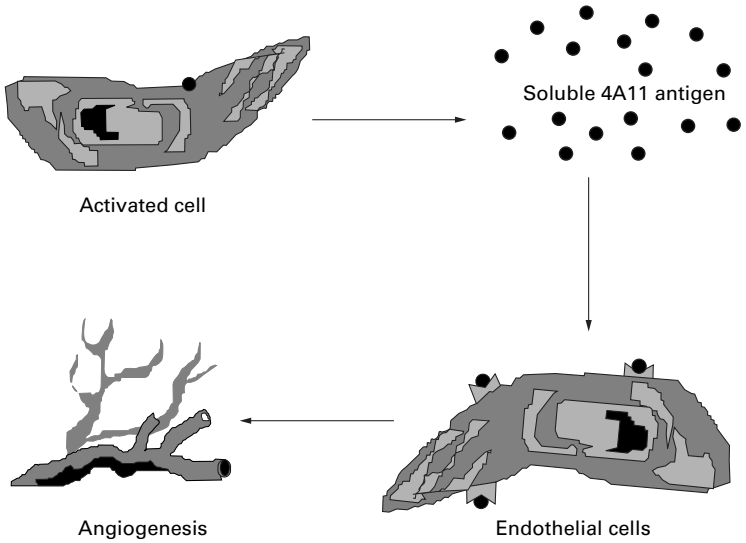

Figure 5 Proposed induction of angiogenesis in RA by soluble 4A11 antigen (Le^y/H).

Glycoconjugates (glycoproteins/glycolipids) have been known for some time to constitute the chemical basis for several blood group systems in humans and to act as adhesion molecules for microbial ligands, though no physiological role has been shown for them until recently.[53] A mAb MIA-15–15, detecting Le^y/Le^x/H, inhibited the motility of tumour cells in vitro.[54] As the potential of tumour cells for invasion is closely associated with motility, which seems to depend on specific glycosylation, it followed that patients bearing lung carcinomas identified by mAb MIA-15–15 had a strikingly worse prognosis than those whose tumours were MIA-15–15 negative.[54] The importance of glycoconjugates in the induction of autoimmunity was recently underscored by the finding that *Helicobacter pylori*, the microorganism involved in gastritis, ulcers, adenocarcinoma, and lymphoma of the stomach, expresses Le^y/Le^x/H, which is also found in gastric mucin.[55 56] Mice bearing hybridomas making *H pylori* induced anti-Le antibodies developed gastritis, pointing to a mechanism by which *H pylori* participates in "molecular mimicry". Hence, antibodies directed against *H pylori* Le^y result in gastritis via an autoimmune reaction directed against gastric mucin Le^y. In diseases such as RA, the inciting agent is unknown. Thus, it is possible that Le^y/H may also trigger a "molecular mimicry" immune reaction in inflammatory angiogenic sites such as RA.

There exists a hypothesis that while endothelium is quiescent for weeks or longer, endothelial cells must also require a mechanism of storage of "preformed" regulators of angiogenesis that are capable of inducing new capillary growth within hours in response to angiogenic stimuli, such as those found in a wound or an inflamed synovial tissue.[57] Despite this hypothesis, with the possible exception of bFGF, examples have not been describe for angiogenesis inducers. The rapid cell surface expression of Le^y/H may fit this paradigm. However, an alternative scenario concerning the regulation of angiogenesis by inducers and inhibitors may be that "structural" mimicry plays a part.

Hence, for instance, a search based on crystal structure revealed that the angiogenic inhibitor endostatin was most homologous to the angiogenic mediator E-selectin (Bjorn Olsen, data presented at the Angiogenesis in Cancer Meeting, Orlando, FL 24–28 January 1998). It is probable that in an RA joint Le^y/H may be stored for expedient use during times of active inflammation and subsequent angiogenesis.

Angiopoietins and ephrins control vascular growth and development

Though as yet not examined in RA, the angiopoietins are increasingly being recognised as modulators of vascular development that may eventually play a part in vascular targeting. Angiopoietin-1 participates in the maturation of blood vessels while angiopoietin-2 is a natural antagonist of angiopoietin-1.[58–60] Interestingly, both angiopoietin-1 and -2 bind the tyrosine kinase receptor Tie2. In vascular development, membrane bound ephrin-B2 marks future arterial cells and its Eph-B4 receptor marks future venous cells.[61 62] If either the angiopoietin-1 gene or the ephrin-B2 gene are deleted in mice, angiogenic remodelling is preturbed.[58 61] A new aspect of angiogenesis has recently been explored. Angiopoeitin-1 overexpressing mice form blood vessels that are not leaky.[58 59 63] This is in sharp contrast with VEGF overexpressing mice, who form leaky vessels. Moreover, overexpression of angiopoietin-1 results in resistance to leakage caused by inflammatory agents in mice. As we learn more about the process of vessel growth and development, it is tempting to speculate that in the future certain types of angiogenic factors may be used to "harden" vessels against the effects of inflammatory mediators.

Angiogenesis inhibitors

PARADIGM OF INHIBITORS RESIDING WITHIN LARGER PROTEINS

The regulation of angiogenesis is likely to result from a delicate balance of angiogenesis inducers and inhibitors (fig 1).[64] Currently, a number of angiogenesis inhibitors have been identified, some of which fit neatly into emerging paradigms. One paradigm mentioned above is that angiostatic activity often resides in portions of larger common proteins that may or may not themselves be angiostatic.[57] Examples of this include many molecules thought to play a part in RA pathogenesis, like thrombospondin, fibronectin and propeptides of type II collagen, platelet factor IV, and fragments of epidermal growth factor. Other mediators fitting this paradigm are: the 29 kDa fragment of fibronectin, the 16 kDa fragment of prolactin, and angiostatin (a fragment of plasminogen), among others.[65–68]

THROMBOSPONDIN AS AN ANGIOGENESIS INHIBITOR

The idea that endothelium is quiescent for long periods of time and yet can be induced to sprout new capillaries in a matter of hours in response to an angiogenic stimulus, suggested that angiogenesis regulators might be stored for expedient use. The first indication of this paradigm was described by Dr Noel Bouck and coworkers, who found that a non-tumorigenic hamster cell line became tumorigenic with a mutation that inactivated a tumour supressor gene.[69] The inhibitory activity was found to be a fragment of the adhesive glycoprotein thrombospondin-1 whose expression was linked to the presence of a tumour suppressor gene. Thrombospondin seems to act via inducing endothelial cell apoptosis.[70] Recently metallospondins have been found using molecular techniques, and are even more potent angiogenic inhibitors than thrombospondin.[71] We were unable to show an effect of inhibiting arthritis or angiogenesis in a rat model of adjuvant induced arthritis.[71 72]

ANGIOSTATIN AS AN ANGIOGENESIS INHIBITOR

Another inhibitor of angiogenesis termed "angiostatin" has been identified in some very elegant studies by Dr Judah Folkman's group.[66 73–77] This factor is a potent inhibitor of tumour growth. Angiostatin is a fragment of the clotting factor plasminogen. Plasminogen itself is not angiostatic. This factor acts by depleting energy required for blood vessel growth by binding ATP-synthase and induces endothelial cell apoptosis by activating focal adhesion kinase.[76–78] The role of angiostatin in RA has not yet been defined.

ENDOSTATIN AS AN ANGIOGENESIS INHIBITOR

Likewise, endostatin, an angiogenesis inhibitor produced by mouse haemangioendothelioma cells, is a fragment of collagen type XVIII.[79] Thus, the fact that abundant components of the circulatory system such as fibrinonectin and plasminogen can be converted to potent angiostatic factors suggests a new form of regulation by proteases, such as serine proteases to specifically release these molecules from their parent molecules. One might envision that in the case of RA, these inhibitors might be present, but downregulated.

Can angiogenesis regulation help us in the treatment of patients with RA?

ANGIOGENESIS INHIBITORS RELEVANT TO THE RHEUMATIC DISEASES

Some of the endogenous and exogenous inhibitors of angiogenesis that have been identified to date include: a cartilage derived factor, troponin, angiostatic corticosteroids, minocycline, fumigillin, thalidomide, choloroquine, sulfapyridine, methotrexate, penicillamine, thiol containing compounds such as gold compounds, taxol, thalidomide, 2-methoxyestradiol, and cyclooxygenase-2 (COX-2) inhibitors.

Interestingly, cartilage is avascular as well as relatively tumour resistant. So, it is reasonable that among the earliest described inhibitors of neovascularisation were cartilage derived inhibitors. Inhibitors of angiogenesis have been purified from various types of cartilage.[80–84] Shark cartilage contains a potent inhibitor of angiogenesis, accounting for its popularity in some circles as an unorthodox treatment for cancer.[85] A cartilage derived inhibitor from bovine scapula has been described that is also an inhibitor of collagenase. Recently, a human cartilage derived angiogenesis inhibitor has been shown to be troponin I, a protein responsible for regulation of muscle contractions.[80 81 86] Like angiostatin, tropinin I acts by binding ATPase,

thus depleting energy needed for blood vessel growth. Regardless of the source, it is intriguing that cartilage, which lies in juxtaposition to the inflamed RA synovium, may hold a key to the understanding of how one might inhibit the angiogenic process in the joint.

Fumagillin is an angiostatic compound discovered as a fungal contaminant on an endothelial culture dish that inhibited endothelial growth.[87–90] Derivatives of this compound termed AGM-1470 or TNP-470 inhibit both angiogenesis and arthritis in rodent models.[90 91] Interestingly, angiogenesis may also play a part in bone formation. TNP-470 inhibited ectopic bone formation induced by bone morphogenetic protein in mice.[92] Fumagillin may be a potential therapeutic agent inhibiting arthritis and angiogenesis in RA.

Several angiogenesis modulating agents have recently been tried in rodent models of arthritis. These include taxol, thalidomide, and 2-methoxyestradiol. Thalidomide is an inhibitor of both angiogenesis, which may account for its teratogenic effects on limb bud formation, and TNFα.[78 93] Thalidomide suppressed rat collagen induced arthritis but not via inhibition of the angiogenic cytokines TNFα or VEGF.[94] Taxol, a chemotherapeutic agent, inhibited collagen induced arthritis in rats and inhibited synovial angiogenesis.[95] 2-methoxyestradiol treatment of collagen induced arthritis in mice resulted in a decrease in arthritis. In vitro, this compound inhibited proliferation of endothelial cells.[96]

Prostaglandins, such as prostaglandin E_2 are potent mediators of angiogenesis.[97] COX-2 inhibitors have recently been released and used for the treatment of RA. Not surprisingly, they have been shown to inhibit angiogenesis mainly in tumour models.[22 98–101] It is quite possible that these modulators are also effective in reducing RA angiogenesis.

Sulfasalazine is commonly used in the treatment of a variety of diseases including RA. The active metabolite of sulfasalazine may be sulfapyridine. In one study, while sulfapyridine inhibited endothelial proliferation, sulfasalazine and 5 aminosalicylic acid metabolites did not.[102] We have recently shown that sulfasalazine and sulfapyridine reduce endothelial proliferation as well as chemotaxis.[103] Sulfapyridine can also inhibit phorbol myristate acetate (PMA) induced endothelial tube formation in vitro. We have also shown that sulfapyridine may mediate this effect by reducing endothelial IL8 production.

Therapeutic considerations

CAN ANGIOGENIC MARKERS HELP GUIDE TREATMENT IN RA?

Recently soluble CD 146, an endothelial cell adhesion molecule involved in leucocyte-endothelial interactions, was found to be upregulated in synovial fluids from patients with RA compared with traumatic injury.[104] Patients with early RA had the highest values of this marker in their synovial fluid. Soluble CD 146 correlated significantly with the degree of morning stiffness, the number of tender joints, and the number of swollen joints.

In papers presented at the ACR meeting VEGF and bFGF were shown to be increased in RA compared with normal serum, especially in early erosive RA and in patients who had antibodies to Sa, an endothelial antigen, as well as to collagen.[105] Serum VEGF is increased in early RA.[106] Early RA synovial fluid VEGF and matrix metalloproteinase (MMP)-9 correlate with each other and with arthroscopic synovitis and vascularity scores.[107] Evidence is mounting that markers of angiogenesis may help us assess early RA.

CAN ANGIOGENIC MARKERS PREDICT RA DISEASE OUTCOME?

Also at the ACR, meeting preliminary data using the Euridiss cohort study indicated that sVCAM-1 and CD 31 can predict disability and radiological changes in RA.[108] Serum VEGF was associated with greater disease activity and soluble selectins were associated with increased disability. Hence, it is tempting to speculate that angiogenic markers may help guide us in RA treatment in the future.

WHAT DOES THE FUTURE HOLD IN ANGIOGENESIS MODULATION?

One might envision vascular targeting strategies. For targeting to be effective, it will be necessary to develop markers to assess the synovial vasculature. In addition to clinical markers of disease activity described above, various imaging techniques are being assessed for evaluating synovial vascularity. One such technique used in rat adjuvant induced arthritis is the use of a radiolabelled E-selectin binding peptide.[109] Some imaging techniques already tried in humans include gadolinium-DTPA enhanced magnetic resonance imaging of RA joints, which correlates with blood vessel density as determined by synovial tissue histology.[110] High resolution ultrasound is also new technique that has been tried for RA joint imaging.[111] Using this technique, the investigators found that inactive versus active RA patients had increased vascularity.

Once strategies are developed for monitoring vascular therapies, and if vascularity is shown to be reflective of disease activity, it is probable that vascular targeting may become a reality. Possible strategies include viral vector targeted antiangiogenic gene treatment, as has been tried in animal models of tumour growth with angiostatin, the VEGF receptor flt-1, and the Tie2 angiopoietin-1 and 2 endothelial receptor.[78 112–114] One may envision strategies like gene treatment with dominant negative VEGFR-1 mutants that prevent VEGF mediated effects on the vasculature. Another therapeutic avenue might be gene treatment with modulation of hypoxia response elements that become selectively activated under hypoxic conditions. Methods aimed at inducing vascular proliferation, perhaps by induction of apoptosis may be another potential strategy to disease modification.

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