Adenoviral transgene delivery provides an approach to identifying important molecular processes in inflammation: evidence for heterogeneity in the requirement for NFkB in tumour necrosis factor production

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Abstract

The success of anti-tumour necrosis factor (TNF) treatment, either using antibodies or soluble receptors, has defined TNF as a major factor of the inflammatory response in rheumatoid arthritis (RA). As a result of this success, much attention has been devoted to understanding the molecular mechanisms by which TNF expression and activity is elicited and controlled. By understanding these pathways, it is hoped that key elements of the molecular pathology associated with RA will be uncovered and, as a result, new targets for therapeutic intervention will be identified. However, studying the cell and molecular biology of model systems for RA, such as primary human macrophages, or tissue from rheumatoid joints may present technical problems. In an attempt to overcome this, we have investigated the use of adenovirus as a means of delivering transgenes by which different intracellular pathways can be modulated and examined. Our data show that adenovirus can be successfully used to efficiently deliver transgenes to primary human macrophages and RA joint tissue. Using a virus encoding IκBα, the natural inhibitor of NFkB, we show that the requirement for the transcription factor is not universal, but is dependent on the nature of the stimulus. Furthermore, while NFkB is of importance for the expression of TNF and other pro-inflammatory cytokines (for example, interleukin 6) and the destructive matrix metalloproteinases, this factor is not required for the expression of anti-inflammatory cytokines interleukin 10 and interleukin 1 receptor antagonist.

The complex nature of the mechanisms controlling TNF expression

As macrophages are the major producers of TNF and other pro-inflammatory mediators in RA and inflammation in general, these cells can be seen as the immediate proximal stage to TNF in the cascade that leads to inflammation (fig 1). Therefore, understanding the cell and molecular biology of these cells in health and disease could help to answer some of the questions presented above. Investigating the molecular signalling mechanisms that control the production of TNF and other pro-inflammatory mediators could lead to the identification of targets for future therapeutic intervention. As many elements of signalling pathways are kinases or other enzymes, this would open up the possibility of screening for, or designing, specific low molecular weight inhibitors that could be orally bioavailable and,

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Inflammatory cascade

Cytokines  MMPs  VEGF  Integrins

Rheumatoid arthritis

Figure 1  The macrophage, TNF, and the inflammatory response in RA. The success of anti-TNF treatments support the view that TNF is important in driving the expression of a number of factors involved in the molecular pathology of RA, including cytokines, matrix metalloproteinases (MMPs), integrins and vascular endothelial growth factor (VEGF). Investigating the molecular biology of the macrophage may identify new targets for treatment that may be more closely related to the nature of the stimulus.

thus, overcome the problems associated with protein-based therapeutics. In addition, we hypothesised that the intracellular mechanisms that regulate TNF production, even in a given cell type such as the macrophage, may not be uniform and vary with the nature of the stimulus. If so, and the discriminating elements of different TNF inducing pathways could be identical, one could envisage that with an appropriate inhibitor TNF production associated with inflammation could be selectively suppressed while sparing other more benign forms of cytokine production (fig 2). Evidence for alternative signalling mechanisms controlling TNF expression has been published suggesting a differential use of the transcription factor, NFκB. However, in these studies, the requirement for NFκB seems to segregate between murine macrophages stimulated with lipopolysaccharide (LPS) that required the factor, and human lymphocyte cell lines, activated with PMA/ionomycin, which showed no requirement for this transcription factor, but used NFAT instead.

Previous studies have shown that the mechanisms controlling TNF production and activity are highly complex (fig 3) with major control mechanisms being applied at gene transcription, by the regulation of transcription factors, and post-transcriptionally and translationally involving the large 3’ un-translated region (UTR) of the TNF mRNA. In addition, TNF is processed post-translationally, being synthesised as a trans-membrane protein that is cleaved from the cell by an ADAM family matrix metalloproteinase, TACE (TNF α converting enzyme). Finally, the biological activity of TNF is controlled by the balance between the concentration of the cytokine and the soluble TNF receptor. However, it must be remembered that the majority of data pertaining to molecular mechanisms controlling TNF production in macrophages, have been derived from studies using LPS as a stimulus and mostly using transformed cell lines of human or murine origin. Because of problems associated with the induction of transgenes (a major approach to signal transduction studies) into primary human monocytes and macrophages, these cell systems have not been so widely explored. Even less studied is RA tissue itself where additional problems, related to the amount of material available and the cellular heterogeneity of the tissue, are present. The potential use of such primary human models are an important consideration, as the stimulus driving TNF production in RA is obscure, the relevance of LPS derived data to the disease is unknown.

Adenovirus provides an efficient means to deliver transgene to macrophages

In an attempt to address some of the problems discussed above, it was felt necessary to develop an efficient means to introduce transgenes into macrophages. Conventional transfection techniques are not very effective in cells of the monocyte-macrophage lineage and even cell lines have proved difficult to transfect with high efficiency.  

To overcome problems with
infection requires cells to express the coxsackie-adenovirus receptor (CAR)\textsuperscript{29} and the αvβ3 or αvβ5 integrins as cofactors for viral entry.\textsuperscript{21–23} Entry of the virus into the cell is rapid and the viral genome is delivered directly to the nucleus (fig 4). However, previous attempts to infect monocytes or macrophages have not been very successful, either requiring high ratios of viral particle per cell, (that is, multiplicity of infection (moi)), or achieving low efficiency of infection.\textsuperscript{21,22} Using an adenoviral vector encoding β-galactosidase (Advβgal), we have observed that deriving macrophages from elutriated human primary blood monocytes, by treating cells with macrophage colony stimulating factor (M-CSF) for 48–72 hours, greatly improves the efficiency of adenoviral infection.\textsuperscript{23} This was associated with the upregulation of αvβ5 integrin expression. Under these circumstances, adenovirus could infect about 95% of cells at moi of 50 (fig 5). Macrophages from RA joints were also highly infectible, without any requirement for M-CSF pretreatment; a moi of 40 of Advβgal resulting in >95% infection (fig 5). The high efficiency of adenoviral infection was of great importance as it precludes any need for separating infected from non-infected cells, an important consideration when addressing the mixed culture system of RA joint cells. It was interesting to note that all the cells in the RA synovium were permissive to adenoviral infection, even the RA joint T cells.\textsuperscript{24} This was a very interesting observation as T cells are normally highly refractory to adenovirus infection.\textsuperscript{21}

Using an IκBα transgene to study the role of NFκB in TNF production in health and disease

The establishment of conditions for effective delivery of transgenes to macrophages made it possible to study signalling pathways in these cells in a more specific way than before. The availability of a natural inhibitor of NFκB, in IκBα, and the previous controversy of the role of this transcription factor in TNF production, provided the ideal system for testing the usefulness of adenoviral vector approaches to the study of intracellular signalling in macrophages. Infection of human primary macrophages with a virus encoding IκBα (AdvIκBα) produced a substantial overexpression of the transgene, compared with uninfected cells or cells infected with an adenovirus containing no insert (Adv0) (fig 6A). Infection with AdvIκBα also resulted in the inhibition of LPS induced NFκB DNA binding activity in the nuclei (fig 6B). Assays of LPS induced TNF production showed 65% inhibition when macrophages were infected with AdvIκBα. In contrast, infection with control virus had no effect on TNF production (fig 7A). NFκB binding sites are also found in the promoters of genes for many other proinflammatory cytokines, for example, IL1 and IL6. The effect of NFκB blockade on the expression of these cytokines, after LPS stimulation, was also assessed. Both IL1 and IL6 were inhibited to a similar extent degree to TNF (fig 7A). Macrophages also produce a number of anti-inflammatory factors, IL1

Figure 3 The complex control of TNF expression may provide many targets for intervention. TNF production is controlled at multiple levels. It is possible that any of these could be the source of novel approaches of blocking TNF production. At present soluble receptors (6) is the basis for the successful anti-TNF approach to RA treatment.

low efficiency, permanently transfected cell lines have been derived from macrophage cell lines. However, this approach would not be applicable in human primary monocytes or macrophages as these cells do not proliferate. Moreover, such a strategy would be of no use for RA joint macrophages where the regulation of TNF production has to be studied in the context of other cells (T cells and fibroblast) with which the macrophage is in contact within the environment of the inflamed joint. Viral vectors can provide an alternative tool for the introduction of transgenes, as they are very effective in delivering their own genes to cells they infect. Of the two most commonly used systems, retroviruses, although relatively simple, suffer from the drawback that successful infection requires cells to be in cycle, effectively ruling out their use in human macrophages. In contrast, adenoviral vectors can infect both dividing and non-dividing cells. Adenoviral

Figure 4 Requirements and efficiency of adenoviral infection. CAR = coxsackie-adenoviral receptor.
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The requirement for NFκB is dependent on the nature of the stimulus

A major question that inspired this study was whether there was heterogeneity in the signalling pathways that are used to induce TNF expression. Macrophages were infected with Adv0 or AdvIβxBo and activated by a variety of stimuli known to induce TNF expression; the yeast product zymosan, anti-CD45 antibodies, phorbol 12-myristate 13-acetate (PMA) and ultraviolet light. PMA and ultraviolet light are both known to induce NFκB activation and the expression of TNF induced by both stimulus was effectively inhibited by the overexpression of IκBα (fig 7B). In contrast, zymosan, or anti-CD45 antibody induced activation of TNF expression was unaffected (fig 7B). We have been unable to demonstrate the activation of NFκB binding activity by either stimulus.25 26 It would seem, therefore, that there is diversity in the intracellular signalling mechanisms inducing TNF synthesis, and that both NFκB dependent (LPS, PMA, ultraviolet light), and NFκB independent (zymosan, anti-CD45) exist. We have also observed that anti-CD45 antibodies induces the activation of phosphatidylinositol 3’ kinase in macrophages and that inhibitors of this enzyme, wortmannin and LY294002, inhibit TNF production.26 In contrast, we have previously shown that LPS stimulation of TNF expression is stimulated by wortmannin and is unaffected by LY294002.27

Adenoviral transgene delivery allows the investigation of signalling mechanisms in RA joint cell cultures

The data described above demonstrate that the requirement for NFκB in TNF production is dependent on the nature of the stimulus. As in a disease like RA, the precise nature of the factor(s) (fig 1) inducing cytokine expression is unknown, such questions can be investigated only by direct examination. To do this, RA joint cell cultures were infected with Adv0 or AdvIβxBo. As for LPS stimulated macrophages, TNF production was inhibited by about 70% (fig 8A), indicating that NFκB is an important factor for TNF expression in RA. Moreover, IL6 production was also greatly diminished to a degree similar to that seen in LPS activated cells. However, the inhibition of IL6 production was more moderate (33%) compared with that of LPS stimulated macrophages. IL8 production was also only moderately effected (about 33%). Studies on the anti-inflammatory cytokines showed no inhibitory effect of IκBα on IL1RA or IL10 expression and, if anything, a slight enhancement of IL11 expression. In contrast, there was a substantial inhibition of p75 soluble TNF receptor production (fig 8A). These data would indicate that NFκB is an excellent therapeutic target for RA, because blockade of this transcription factor provides the ideal result, inhibiting the expression of key proinflammatory cytokines, while sparing the major anti-inflammatory mechanisms.

The studies were also extended to investigate the expression of MMPs production by the joint cell cultures. These enzymes are generally considered to be the major players in the

![Figure 5](http://ard.bmj.com/)

Efficient infection of macrophages from the periphery and RA joint tissue. Macrophages were derived from peripheral blood monocytes by two days pretreatment with 100 ng/ml M-CSF, as described previously.23 RA joint tissues were obtained from the synovium from patients with RA undergoing surgery.23 Peripheral blood M-CSF macrophages, or RA joint cell cultures were infected with an adenovirus encoding β-galactosidase (Advβ-gal), kindly provided by Drs Woods and Byrnes, Oxford, UK) at a moi of 50 or 40 respectively. After 48 hours, cells were analysed for β-galactosidase expression by FACS. For RA joint cells, immunofluorescence with anti-CD14 antibodies was used to identify the macrophage population.23

![Figure 6](http://ard.bmj.com/)

Expression of an adenovirus delivered IκBα transgene in macrophages inhibits NFκB activation. (A) Peripheral blood M-CSF macrophages were either uninfected, or infected with an IκBα encoding adenovirus (AdvIκBα), or control virus Adv0 at moi 40. After 48 hours cells were lysed and cytosolic extracts were examined for IκBα expression by immunoblotting. (B) Cells were either uninfected, or infected with AdvIκBα at moi 40. After 48 hours the cells were activated with LPS (10 ng/ml) for 30 minutes followed by nuclear extraction. Nuclear NFκB DNA binding activity was analysed by EMSA.

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cartilage destruction in RA. The expression of both MMP-1 and MMP-3 (collagenase-1 and stromelysin) were inhibited by >60% on AdvIβBα infection (fig 8B). The expression of MMP-13 was less affected (50%). These data would suggest a role for NFκB in the expression of these MMPs. However, a somewhat surprising finding was the observation that the inhibition of NFκB had no effect on the expression of the tissue inhibitor of metalloproteinase 1 (TIMP-1). As for the cytokines, blockade of NFκB would seem to provide the optimum result: inhibition of all three of these destructive enzymes, but no effect on their major inhibitor.

Summary
These studies have demonstrated the potential of using adenoviral vectors for the study of macrophage function in health and disease. The heterogeneous nature of mechanisms controlling TNF production has been demonstrated, with the nature of the stimulus determining the requirement for NFκB. This result has major implications for future approaches to understanding the mechanisms controlling TNF production in different circumstances. Simple cell models may no longer be sufficient to determine or validate the key signalling pathways in a disease process. Adenoviral vectors can also be used in diseased tissue cultures, like RA synovial cells. In this system, we reproduced the findings that TNF and other proinflammatory cytokines were NFκB dependent, but that IL1RA, IL10 and IL11 were not. Equally importantly, several destructive MMPs were NFκB dependent, but their major endogenous inhibitor was not. These results point out NFκB as an important therapeutic target in RA. Therefore, the development and use of more physiologically relevant systems, and the required tools to investigate them, seem necessary for the future.
identification of the key molecular mechanisms driving inflammation.


