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REVIEW

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Anti-inflammatory therapies: application of molecular biology techniques in intensive care medicine

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Introduction

Intensive care has emerged as a new branch of medicine over the last two decades, providing support and applying interventions specific to the needs of critically ill patients. Its rapid growth has occurred in parallel with the rapid development of molecular biology, a new branch of science which has greatly advanced our knowledge of the pathogenesis of many human diseases and now stands poised to have a major impact on the care of the critically ill. Molecular biology techniques, such as the polymerase chain reaction (PCR), differential display [1], transgenic models [2] and many others are helping to increase our understanding of the pathophysiology of diseases and to develop very sensitive and rapid diagnostic tests [3, 4]. Molecular biology is also providing us with new therapeutic targets and the potential to approach the treatment of diseases from entirely new directions. Unfortunately in this context, the exciting potential of biotechnology has been disappointing to date, with a number of negative clinical trials of potentially exciting drugs [5–8]. There are a number of reasons for these failures, including the difficulty of identifying patients who would benefit from such therapies, the targeting of only one or two molecules in complicated inflammatory cascades, and our lack of understanding of fundamental disease processes.

The goal of this review is to describe a number of approaches and techniques that are relevant to the critically ill and that are based on principles of molecular biology. Rather than simply providing a generic overview of basic molecular technology, we will use the acute inflammatory response, and specifically the role of cytokines, as a model to examine the present and future potential of molecular biology and to indicate how these approaches might overcome the problems inherent in previous clinical trials. However, we do not wish to imply that cytokines are the only, or even the most important, targets in the care of the critically ill; here they serve only as a model system for discussion.

Cytokines and acute inflammatory reactions

A major cause of morbidity and mortality in the critical care setting is the development of multiple organ dysfunction syndrome (MODS) [9]. In this regard, many diverse conditions (e.g., sepsis, multiple trauma, pancreatitis), result in the generalized intravascular activation of inflammatory cells with endothelial and/or parenchymal injury. Evidence indicates increasingly that the pathophysiology of the host inflammatory response is mediated by cytokines, which are extracellular signalling proteins secreted by effector cells. These molecules have as their primary function the ability to modify the behaviour of other closely adjacent cells [10]. Advances in cytokine biology have paved the way for innovative approaches into the treatment of acute respiratory distress syndrome (ARDS), septic shock and MODS. Several immunotherapies, for example, have been subjected to clinical trials during the last decade; unfortunately, none with any great success.

Newly developed molecular technologies, such as molecular cloning, molecular engineering and gene transfer have been employed to improve the efficiency of these therapies.

Over the last decade, elegant studies of the basic biological characteristics of inflammation and tissue injury have implicated cytokine-mediated vascular and tissue injury in the pathogenesis of a wide variety of immune and inflammatory clinical disorders, including sepsis, shock, ARDS and MODS. If the intravascular inflammatory response becomes uncontrolled or excessive, the vascular endothelium will be damaged, leading to distant organ injury [11]. Regardless of the underlying insult, the response of the host is to initiate an inflammatory response, resulting in the release of numerous mediators, in particular the cytokines, into the circulation [9]. Cytokines are generally divided into pro- and anti-inflammatory mediators, which have opposite effects in mediating inflammatory reactions. In patients with septic or traumatic shock, tumor necrosis factor (TNF), interleukin (IL) 1 and, to a lesser extent, other cytokines appear to potentiate organ injury by inducing a change in endothelial cells from a non-inflammatory to a pro-inflammatory phenotype. Activated endothelial cells express surface receptors, such as E-selectin, P-selectin, and intercellular adhesion molecule-1 (ICAM-1), that promote leukocyte adherence. These pro-inflammatory cells also express and/or secrete factors, such as IL-6, IL-8, and platelet-activating factor, that attract or activate other cells which further fuel the inflammatory response. The shift in endothelial phenotype ultimately results in focal microvascular thrombosis and leukocyte-mediated endothelial injury. Similarly, inflammatory factors, such as complement fragments and eicosanoids, activate circulating neutrophils and upregulate complement receptor expression, thereby increasing neutrophil adhesiveness and consequently neutrophil endothelial binding [11].

Because cytokines are potent bioactive molecules, it is not surprising that their production is tightly regulated at several steps [12]. Cytokines exert their effects by binding to their membrane receptors and activating intracellular signal transduction pathways, leading to alterations of targeted cells. Each of these steps may be a target for therapeutic manipulations. Important regulatory control on cytokine action is exerted by cytokine-neutralizing molecules that are classified as soluble receptors and receptor antagonists of cytokines [13]. The counterbalance between the plasma concentration of a cytokine and its neutralizing molecules determines the expression of bioactivity of that particular cytokine. Cytokine activity can be regulated in a number of ways by these cytokine-binding proteins that are present in the micro-environment. For example, in the case of TNFα, the circulating TNFα binding proteins may bind to TNFα, limiting access of TNFα to the membrane-bound receptor and hence acting to neutralize TNFα. However, it is also possible that this intravascular binding of TNFα may prevent its degradation and thus prolong TNFα activity, albeit at lower levels [14]. Other binding molecules, including several acute phase proteins (e.g., α2-macroglobulin), may influence the biological activity of several growth factors and cytokines, thus also playing an important role in attenuating or modifying the kinetics of circulating cytokines [15].

In this review, we discuss the application of molecular biological techniques in the development of the anti-inflammatory therapies under four categories: 1) neutralizing antibodies to block cytokine-inducers, pro-inflammatory cytokines and their cell-surface receptors, 2) blocking the interaction between cytokines and cell-surface receptors with naturally expressed molecules, 3) enhancing protective activities in target cells and 4) the intracellular blockade of cytokine-induced responses.

Neutralizing antibodies

Polyclonal antiserum, monoclonal antibodies, and antibody fragments have all been developed to mitigate acute inflammatory reactions, by blocking inducers of cytokines such as endotoxin, by neutralising pro-inflammatory cytokines, or by neutralising their cellular membrane receptors (Fig. 1). The availability of commercial quantities of high-affinity anticytokine antibodies has made their therapeutic use in humans possible.

Antiserum and polyclonal antibodies

Because of the important role that endotoxin plays in the pathogenesis of septic shock, there has been considerable interest in the evaluation of antibody therapy directed against endotoxin. The goal of immunotherapy in the treatment of serious Gram-negative infections is to prevent or attenuate the adverse effects of endotoxin on the central and systemic vasculature as well as the effects of endotoxin on lung and systemic organ injury [9]. The first clinical study examining the effects of anti-endotoxin antibodies on human sepsis was performed with polyclonal J5 antiserum. This antiserum was produced by injecting the heat-killed mutant of the *E. coli* J5 bacterial strain into human volunteers and extracting the resultant polyclonal serum. Preliminary experiments suggested that this antiserum reduced mortality by approximately 45 % in patients with Gram-negative bacteremia [16]. It was also used prophylactically in high-risk surgical patients and protected them from developing septic shock in the postoperative period [17]. However, there are several serious drawbacks to using human antiserum or polyclonal antibodies, including

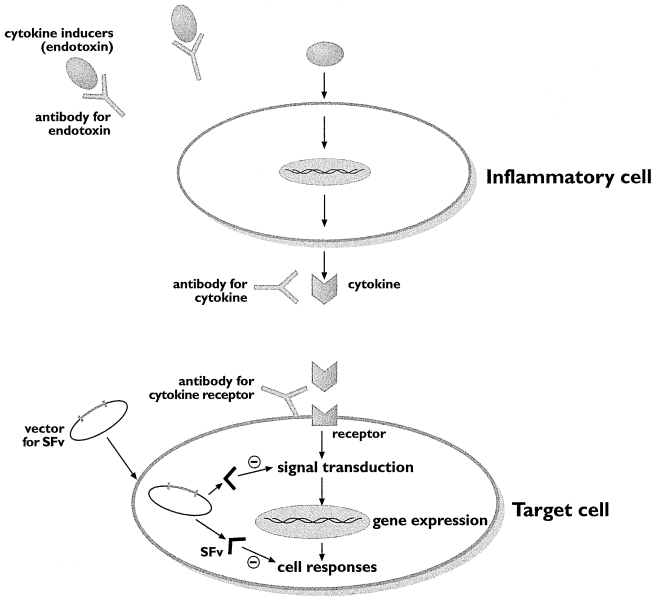

Fig. 1 A schematic illustration of the principles of antibodies against endotoxin, cytokines and their receptors in the anti-inflammatory treatment. Neutralizing antibodies, either in their native form, or humanized by molecular engineering, or modified single-chain domains, may selectively block cytokine inducers, such as endotoxin, pro-inflammatory cytokines and their cell-surface receptors. A new method, intracellular antibody targeting, is also illustrated as a potential therapeutic approach (SFv: Single-chain antibody fragment, see detail in the text)

mild toxicity in the vaccinated donors, the risk of transmitting infection from the donors to the recipients of the antibody and difficulty in standardizing the effectiveness of the antiserum among donors [9].

Monoclonal antibodies

To circumvent the problems surrounding the use of a pooled human antiserum, monoclonal antibodies have been developed. These are the secretory products of hybridomas, cells fused between antibody-secreting B lymphocytes (from immunized rodent or human) and a myeloma cell line. Generally, it is difficult to produce human monoclonal antibodies, because human B cells immortalized by fusion with a myeloma cell are usually unstable and antibody yields tend to be low [18]. One of the first studies to use this approach examined a murine IgM anti-endotoxin monoclonal antibody (E5) in patients with Gram-negative sepsis [6]. Human anti-mouse antibodies were observed in 47% of patients treated with E5 associated with a modest improvement in survival, as well as more frequent resolution of individual organ failures, was observed [6]. The second trial using E5, conducted in 847 patients with Gram-negative sepsis who were not in refractory shock, observed a trend towards improved survival in a subgroup of 139 patients who developed or presented with evidence of major organ failure [19].

Ziegler and co-workers [5] developed an IgM mouse-human hybrid anti-endotoxin monoclonal antibody (HA-1A) and used it in 543 patients with sepsis. There was no significant difference in overall mortality rates among the recipients of placebo and those given HA-1A. In a subgroup of patients with Gram-negative bacteremia, HA-1A reduced mortality among patients with or without shock [5]. However, contradictory data also exist regarding the effects of the antibody [20], in that a second multicentre, placebo-controlled clinical trial was stopped early due to a higher mortality observed in the group receiving HA-1A [21]. Cross-reactivity between HA-1A and anti-mouse IgG antiserum has also been reported [22].

TNFα is one of the principal proximal mediators of sepsis inducing other cytokines, including IL-1, IL-6, and IL-8. Plasma concentrations of TNFα are increased early in the course of septic shock and correlate with the severity of physiological derangement [13]. Thus, blocking TNFα action is a logical target to interrupt the cytokine cascade. An initial study using a monoclonal antibody (CB0006) to human recombinant TNFα in 14 patients with severe septic shock demonstrated that the treatment appeared to be safe and was associated with some improvement in hypotension [23]. A transient improvement in ventricular function and arterial oxygenation was observed with the administration of the anti-TNFα to 10 patients with septic shock [24]. A phase II multicentre trail of the anti-TNFα antibody in 80 patients with severe sepsis showed the treatment to be safe but not efficacious, except in seven patients who, identified retrospectively, had high plasma concentrations of TNFα on entry into the study. In this study, 98% of individuals treated with the murine antibody developed antimurine antibodies [25]. Alternatively, neutralizing antibodies can be used to block the binding of cytokine to their cell-surface receptors [13]. For example, monoclonal antibodies to the IL-1 receptor have been used to protect animals from lethal endotoxemia [26].

It is important to note that in sepsis and acute inflammatory injuries, TNF is often viewed as a pathogenic mediator, but in other disorders the biological activities of TNF may enhance immunological function and protect the host [27]. For example, TNFα in lower concentrations may be useful in suppressing granulomatous infections (e.g., tuberculosis, leishmaniasis) [27] and infections caused by *Pneumocystis carinii* [28]. It is only in conditions such as sepsis, where TNFα concentrations are no longer compartmentalized or there is "uncontrolled" overexpression, that TNFα may be damaging. This risk/benefit ratio must be considered in therapy with anti-TNF antibodies [29].

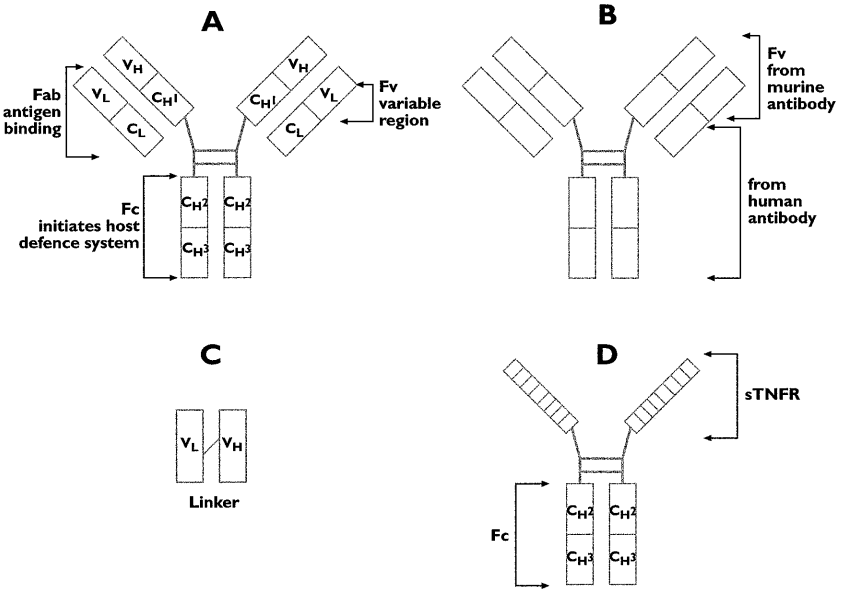

Fig. 2 Schematic structures of an antibody and modified antibodies with molecular engineering technology. (**A**) A schematic structure of a native form antibody. Its antigen-binding site comprises two variable regions and two constant regions, one each from the heave and light chains. Fc region comprises two constant regions from the heavy chain. (**B**) A "humanized" antibody. Its antigen-binding site from antibodies raised in mice is joined with human IgG to make a chimeric antibody, which can reduce immunogenicity and enhance effector functions. (**C**) Single-chain antibody fragment (SFv) is a recombinant polypeptide composed of variable chains covalently held together via a linker peptide. It may possess specific binding affinities equivalent to the parent antibodies. (**D**) Construct of chimeric molecule of soluble TNF receptor (sTNFR) and heavy chain of IgG antibody

Antibody engineering

There have been extensive and extremely productive recent investigations into the structure and function of antibodies (Fig. 2 A) that may help overcome some of the difficulties inherent in the clinical studies described above [30]. It is now possible to amplify and isolate most of the antibody variable genes from a diverse population of human or murine B cells, thereby generating an antibody gene library. The fine structure of rodent antibodies differs significantly from that of human antibodies. In many cases the Fc portion of rodent antibodies cannot be recognized by human Fc receptors and complement proteins, resulting in a failure to initiate human defence mechanisms. Mouse monoclonal antibodies can also elicit a strong antimurine protein immune response that greatly reduces their circulating half-life [31]. This immunological response is associated with decreased biological activity on re-administration of a murine monoclonal antibody, as well as adverse effects such as anaphylaxis and serum sickness.

To ameliorate these problems, chimeric (part human, part mouse) monoclonal antibodies have been developed using the technique of antibody engineering. By isolating the genes that encode the hypervariable (V) and constant (C) regions of rodent monoclonal antibodies and the C regions of human immunoglobulins, genetic constructs can be created and transfected back into murine myeloma cell lines. These "transfectomas" secrete "humanized" monoclonal antibodies that exhibit the binding capacity of the original murine monoclonal antibody, coupled with the C regions of a human immunoglobulin molecule that leads to a reduced human antimurine antibody response (Fig. 2 B) [32, 33]. Chimerization also enhances the effector functions of these antibodies. Structural analysis of antibody-antigen complexes shows that the antigen-binding surface of the antibody is formed by six hypervariable loops of amino acids called complementarity determining regions [18]. These loops are mounted on relatively constant framework regions and by genetic manipulation can be transplanted from a rodent antibody on to a human antibody framework. This approach can further reduce the immunogenic response that may be generated against the V regions of chimeric monoclonal antibodies [34].

Single-chain antibodies

The Fv fragment of an antibody molecule, composed of the variable regions of light and heavy chains (V_L and V_H), is the minimal structure required to retain the binding site specificity of the parent antibody [35]. PCR technology has been used to directly clone the V_L and V_H specific moieties from hybridoma cell lines [35]. Incorporation of the sequences encoding V_L and V_H domains into a single gene (SFv) can dramatically simplify antibody engineering (Fig. 2 C). Active SFv proteins that bind haptens, proteins, receptors or tumor antigens have been shown to have specific binding affinities equivalent to those of the parent monoclonal antibodies [30, 35]. Advances in the design of single-chain antibodies and antigen binding proteins promise increased utility and new capabilities for these molecules.

An SFv antibody chain against TNF receptor has been produced by molecular cloning. V_H and V_L encoding sequences for TNF receptor binding sites were isolated by PCR from the murine hybridoma cell line,

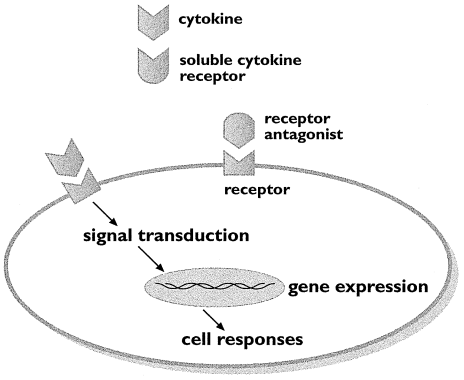

Fig. 3 The potential roles of soluble cytokine receptor and cytokine receptor antagonist in anti-inflammatory therapies. Soluble cytokine receptors are homologous to the extracellular binding portion of the corresponding cytokine receptors that can bind to cytokines in the circulation and prevent their binding to the cell-surface receptors. Cytokine receptor antagonists are cytokine-like molecules that bind to cytokine receptors but do not invoke responses

H398, then cloned into an SFv expression vector and expressed in *E. coli.* In an in vivo assay, the SFv is an efficient inhibitor of TNF-mediated cytotoxicity. Because it possesses the high affinity TNF receptor-binding and receptor-antagonistic activity, but is expected to be less antigenic in man, it may become a valuable tool for the development of novel therapeutic reagents against TNF-mediated inflammatory reactions [36].

Human monoclonal antibody cocktail

In vitro gene amplification and genetic engineering of bacteriophages have produced large antibody gene libraries and facilitated the large scale production of human monoclonal antibodies with high specificity [31]. The natural immune response is polyclonal and thus a cocktail of monoclonal antibodies raised against particular antigens may be more effective than a single antibody. It should be possible to isolate multiple human antibodies to any antigen from a single library of sufficient size. For example, a large number of human antibodies against HIV have been generated from the bone marrow of a person infected with HIV [37]. In time, the whole process may become automated and such libraries may allow the generation of reproducible "polyclonal human antiserum" against any antigen [31].

Naturally expressed endogenous cytokine inhibitors

There are naturally occurring molecules that mediate or modulate cytokine activity. One such class of molecules contains soluble cytokine-binding proteins that represent secreted or proteinase-cleaved cell-surface cytokine receptors [15]. Soluble cytokine receptors are homologous to the extracellular binding portion of the corresponding cytokine receptors and can bind to cytokines in the circulation and prevent binding to intact cell-surface receptors (Fig. 3). Another group of naturally expressed cytokine inhibitors function as cytokine receptor antagonists. They are cytokine-like molecules that bind to cytokine receptors but do not invoke a response (Fig. 3). Thus, the net biological effect of host inflammatory mediators probably depends on their presence relative to specific and non-specific inhibitors [20]. Many questions remain with respect to the application of these naturally expressed proteins, such as the difficulty of identifying patients who might benefit from this approach and the potential risk of compromising the cytokine-mediated host defense. However, the discovery of these endogenous cytokine inhibitors opens a new approach for anti-inflammatory therapies. Because the original source of these inhibitors is human, application of these proteins, produced in large scale by bioengineering techniques, could reduce the risk of immune responses.

Soluble cytokine receptors

Several specific soluble cytokine-binding proteins have been identified that include the soluble receptors for IL-1 [38], IL-2 [39], IL-4 [40], IL-6 [41], granulocyte-macrophage colony-stimulating factor (GM-CSF) [42], and TNFα [43–46]. Two TNF soluble receptors, termed sTNF-R55 and sTNF-R75, have been identified that possess sequence homology to the 55-kDa receptor from the surface of polymorphonuclear leukocytes and to 75-kDa receptor from the surface of epithelial cells, respectively. Both types of TNF soluble receptors may be shed from the cell membrane [45]. The TNF receptor expressed by activated T cells undergoes proteolytic cleavage at the cell surface, releasing a 40-kDa soluble TNF-binding protein [46]. In vivo studies suggest the possible utility of this approach in that mice were protected against endotoxic shock by blocking TNFα from binding to cell-surface TNF receptors [47]. The particular receptor that is targeted may have an important impact on the efficacy of therapy. For example, the protective effect of the 55- but not the 75-kDa TNF soluble receptor-IgG fusion protein was shown in an animal model of Gram-negative sepsis [48]. A phase II randomized, double-blind, placebo-controlled multicentre clinical trial has been initiated [49].

Cell-surface cytokine receptor antagonists

The IL-1 receptor antagonist (IL-1ra) is a molecule that binds to the IL-1 receptor, but does not initiate IL-1 signal transduction [50]. Its amino acid sequence shares

26% homology with IL-1β and 19% homology with IL-1α [51]. Treatment with IL-1ra prevented neutrophilic lung inflammation in rats treated with endotoxin or recombinant IL-1 [52]. Human IL-1ra blocked IL-1β-induced hypotension and leukopenia [53] and hemodynamic, hematological, and pulmonary effects of *E. coli* infusion in rabbits [54]. Fisher and co-workers [7] initially reported a dose-dependent, 28-day survival benefit in 99 patients with sepsis syndrome treated with an increasing dose of IL-1ra (from 17 to 133 mg/h) administered as a continuous infusion. Based on these encouraging data, IL-1ra was used in a larger, more definitive phase III trial in 893 patients with sepsis syndrome. There was no significant increase in survival for the IL-1ra treated groups compared to placebo-treated groups, although in a post hoc analysis there was a modest reduction in mortality in a subset of 595 high-risk patients [55].

Designer molecules

Molecular engineering has also been used to enhance the function of naturally expressed anti-inflammatory mediators. Several bivalent fusion proteins have been constructed with soluble TNF receptor attached to the hinge region of the heavy chains of mouse IgG1 [56], human IgG1 [57] or IgG3 [58] (Fig. 2 D). These chimeric molecules bind with greater affinity to the natural trimeric forms of TNFα and appear to have enhanced neutralizing capacity. The dimeric soluble TNF receptor-Fc molecule has been shown to be substantially more efficacious than monomeric soluble TNF receptor in protecting mice from lethal endotoxemia [14] and is currently being investigated in a phase II clinical trial.

Enhanced anti-inflammatory responses using gene transfer

Gene therapy is a therapeutic approach by which a recombinant gene is introduced into the cells of patients to treat an inherited or acquired disorder. These genes can then synthesize a missing or defective gene product in vivo [59]. The use of genes as drugs, or in combination with drug therapy, to enhance anti-inflammatory responses has tremendous potential in the critical care area.

Methods for gene transfer

The first step in the use of gene therapy is to introduce the gene of interest into the target cell. Two general categories of gene transfer vectors have been developed: viral and non-viral. Both approaches have been used to deliver therapeutic genes to treat acute inflammatory reactions. Viral vectors, either from retrovirus or adenovirus, are designed to exploit the efficient cellular entry characteristics of various virions to accomplish delivery of a therapeutic gene. To produce a replication-deficient virus, that is a virus that lacks the genetic machinery necessary for the production of additional viruses, the therapeutic gene is first inserted into a shuttle DNA construct that contains some portion of the viral genome but has one or more of the viral genes necessary for production of replication-competent virus deleted. Non-viral approaches, such as liposomes and molecular conjugates, have been designed to exploit endogenous cellular entry mechanisms to permit efficient delivery of the therapeutic gene [60]. Liposomes are artificial lipid bilayers designed to translocate drugs or nucleic acids into the cell cytosol via cell-membrane fusion or endocytosis [61–63]. Each vector system has its advantages and disadvantages, depending upon the target organs, cell proliferation status and many other factors. The fundamental problems with current gene transfer systems have recently been reviewed [60].

Strategies for anti-inflammatory gene therapies

Genes encoding anti-inflammatory cytokines, antioxidant enzymes, antiproteases and other protective proteins have been identified to treat acute injury in the lung and other organs [60, 64] (Table 1). The most direct approach would be to administer systemically anti-inflammatory cytokines (e.g., IL-10, G-CSF) or protective proteins directly. However, there are several advantages of giving therapeutic genes rather than proteins: 1) gene transfer is usually less expensive than giving purified or recombinant proteins, 2) depending upon the delivery systems chosen, gene transfer may have less risk of antigenicity, 3) with modified vectors, gene transfer could be cell type specific and 4) gene transfer may deliver genes encoding intracellular proteins.

Anti-inflammatory cytokines, such as transforming growth factor-β, IL-4, IL-10, IL-1ra and G-CSF, may modulate the inflammatory response and promote the repair of damaged tissue. For example, IL-10 is a homodimeric peptide with a wide spectrum of anti-inflammatory activity. A replication-deficient adenoviral vector expressing murine IL-10 has recently been constructed. Intramuscular injection of this vector in mice led to significant and prolonged circulating levels of bioactive IL-10 and markedly suppressed endotoxin-induced TNFα and IL-6 upregulation [65]. An adenoviral vector encoding a TNFα soluble receptor provided protection against endotoxic shock in mice and modified the inflammatory responses induced by *Pneumocystis carinii* and intratracheal silica [66].

Aggressive reactive oxygen species are generated by neutrophils and other inflammatory cells during the

Table 1 Example of gene transfers in anti-inflammatory therapies

Rationale	Gene(s)	References
Anti-inflammatory cytokines	IL-10	65
Cytokine inhibitors	Soluble TNFα receptor	66
Antioxidant enzymes	Catalase	67
Antiproteases	α-1 antitrypsin	68
Metabolic enzymes	Prostaglandin G/H synthase	69
	Nitric oxide synthase	70
Surfactant apoproteins	SP-A	71
	SP-B	71, 72
Stress tolerance	Heat shock proteins	73

acute inflammatory response. The resultant oxidant stress is thought to be important in acute lung injury and other organ injury associated with shock and sepsis [74]. An adenovirus vector has been used for direct in situ delivery of the catalase gene to the pulmonary vascular endothelium, to provide augmented vascular antioxidant defence in the context of ARDS and other pulmonary vascular diseases [67].

Cationic liposomes have been used to deliver the prostaglandin G/H synthase gene into the pulmonary artery, which protected rabbit lung from endotoxin-induced injury [69]. The nitric oxide synthase gene has been delivered to endothelial cells via liposomes, and inhibited neointimal vascular lesions [70]. Proteolysis associated with the acute inflammatory response is thought to be an important part of the pathogenesis of acute lung injury. Increasing production of potent antiproteases, such as α-1 antitrypsin, in lung cells at the site of injury might be especially effective in preventing injury associated with inflammation [64, 68].

Cells primed by an initial, non-lethal stress, such as a mild increase in temperature or a low dose of sodium arsenite, become more resistant to a second stress that otherwise should be lethal. This phenomenon has been termed stress tolerance [73], and has been associated with the expression of heat shock porteins (HSPs), especially HSP72. Cells transfected with the HSP72 gene, under the control of a constitutive promoter, were shown to be more resistant to thermal stress than non-transfected cells [75]. Additionally, cells expressing HSP72 were found to be resistant to the cytotoxic effects of TNFα and TNFβ [76–78]. The induction of the heat-shock gene by either total-body hyperthermia or administration of sodium arsenite or ethanol protected rodents from lethal doses of endotoxin [79–81] and from sepsis [82, 83]. The induction of HSPs has been correlated with lung protection from intratracheal instillation of phospholipase A_2 [84]. Thus, the expression of HSPs used prophylactically may be a potential treatment to protect and preserve cellular function in an individual, or within a particular organ [73].

Intracellular blockade of cytokine synthesis or cytokine-induced cellular responses

Many of the approaches designed to inactivate inflammatory proteins involve the development of compounds that bind to cytokines or their receptors. Another approach that acts at the source is specifically to prevent the synthesis of pro-inflammatory cytokines or cytokine-initiated intracellular signalling. To achieve this objective, nuclei acids can be considered for therapeutic uses, either to interfere with the function of specific nucleic acids or to bind specific proteins [85]. There are three principal types of application of this methodology: a) triplex forming (anti-gene) compounds, which bind specifically to double-strand DNA and prevent transcription, b) antiprotein oligos or "aptamers", which bind to proteins and exert a biologic effect and c) antisense oligos, which bind specifically to a target mRNA and prevent translation [85, 86]. In this article, we will discuss antisense technology only. Using this approach, it is possible selectively to inhibit the synthesis of pro-inflammatory cytokines or intercellular adhesion molecules related to acute inflammation (Fig. 4) or to inhibit cytokine-induced responsiveness by blocking the expression of specific signalling proteins (Fig. 5). In addition, a recently developed molecular technique, intracellular immunology (Fig. 1) will be briefly mentioned.

Antisense oligodeoxynucleotides

In double-stranded DNA, the nucleotide sequence containing the information that is translated into the amino acid sequence of the protein is called the *sense* strand. Its complementary nucleotide sequence is called the *antisense* strand [87]. A short stretch of nucleotides of the antisense-strand molecule can by synthesized and delivered to cells, which can bind to the sense strand of DNA or mRNA, to "fool" the cellular machinery and hence block protein production. Antisense can be delivered either as synthetic DNA oligodeoxynucleotides (ODNs) added to cells from the outside, or as antisense RNA

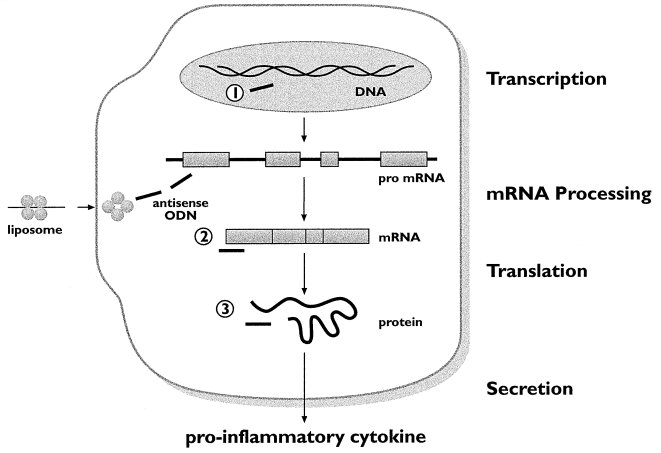

Fig. 4 Potential sites for antisense oligodeoxynucleotides (ODNs) to block the synthesis of pro-inflammatory cytokines. Antisense ODNs can be delivered into the target cells via liposomes. They may selectively inhibit DNA transcription (1), block translation from mRNA to protein (2), or interfere with protein functions (3). Antisenses may also block export of functional transcripts from nucleus, affect the splicing of pre-mRNA or alter the stability of mRNA and ribosome assembly (not shown)

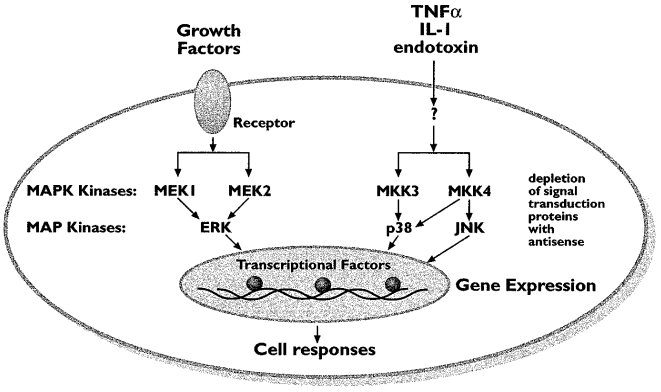

Fig. 5 Potential application of antisense ODNs in blocking endotoxin- or pro-inflammatory cytokine-induced intracellular signal transduction. Growth factor-initiated signals may be transmitted through MAP kinases such as ERKs. In contrast, endotoxin, TNFα and IL-1 may activate JNK and p38 MAPK. Treatment of cells with antisense ODNs to attenuate the expression of these signal transduction proteins may selectively inhibit endotoxin- or pro-inflammatory cytokine-initiated cellular responses

transcripts generated by expression vectors in transfected cells using the cells own transcriptional machinery. Because the latter approach has a number of problems in terms of efficacy and safety, the more common approach is to use antisense ODNs. It is critical that these oligos bind to the specific RNA or DNA that is to be blocked, and this requires a certain minimum number of nucleotides, usually 15–20. In principles, a suitable oligo should be able to interfere, in a sequence-specific manner, with processes such as the translation of mRNA into protein [88]. Sequence-specific inhibition of mammalian mRNA translation in tissue culture by oligos has been achieved. However, the exact mechanisms by which antisense oligos interfere with the gene expression, translation and functions of proteins are unknown. Several hypotheses have been proposed [89]. For example, antisense can target many points in the gene expression pathway, including the transcription factor binding site, transcription bubbles, intron splice sites, etc. (Fig. 4).

The major difficulties inherent in this approach are the development of the appropriate oligo, delivery to the requisite cells and ensuring specific and efficient blockade. DNA oligomer modulation of gene expression is transient. The relative stability and uptake characteristics of many modified oligos, such as phosphorothioates and methylphosphonates, have been well characterized [90]. Extensive efforst to develop and apply antisense oligos for the therapy of a number of diseases are being made. Oligos can be delivered systemically to animals as naked DNA, in liposomes or by conjugation to other molecules, permitting more efficient uptake by cells. Phase I clinical trails designed to evaluate the toxicity of ODNs in cancer patients have commenced [88]. The requirements and experimental strategies for using antisense oligos as therapeutic agents have been the subject of several reviews [88, 91, 92].

Antisense against pro-inflammatory cytokines

Although at present the use of cytokine antisense primarily represents a tool for dissecting the function of cytokines in vitro, the therapeutic application of antisense technology has been suggested by studies in which the inhibition of inflammatory cytokines has been produced in vivo [93–95]. There is increasing evidence to suggest that antisense oligos can produce local, regional and systemic effects at non-toxic doses in vivo [96].

Antisense have been used extensively to study the role of cytokines in controlling cell proliferation and differentiation [93, 97] (Table 2). To enhance the efficacy of antisense delivery to macrophages to block TNFα synthesis, several chemical modifications have been attempted. For example, morpholino-modified antisense oligos directed against TNFα mRNA at different sites were tested [103]. To achieve macrophage-specific oligonucleotide delivery, a molecular conjugate consisting of mannosylated polylysine that exploits endocytosis via the macrophage mannose receptor was developed. It effectively inhibited silica-induced TNFα in alveolar macrophages [104]. Effect of IL-1 in mice was inhibited by systemic administration of antisense oligos against IL-1 receptor [106].

Several cytokines have been shown to be autocrine growth factors or growth regulators for various subtypes

Table 2 Example of sequence-specific inhibition of mRNAs encoding inflammation-related cytokines and intercellular adhesion molecule by oligodeoxynucleotides: potential targets of anti-inflammatory therapies

Gene product	References
IL-1	98
IL-1α	98
IL-1β	99
IL-2	100
IL-3	101
TNF-α	102–104
ICAM-1	94, 95, 100
INFγ	105
IL-1 receptor	106

of lymphocytes or macrophages. For example, T helper cells have been divided into two subsets on the basis of their lymphokine secretion and requirement for growth. The Th1 subset produces IL-2 upon antigen or lectin stimulation, whereas the Th2 subset produces IL-4. Antisense oligos complementary to IL-2 inhibited the proliferation of the Th1 clone and had no effect on the Th2 clone. By contrast, antisense IL-4 blocked the proliferation of Th2 clone and not that of the Th1 clone, indicating an autocrine mechanism of the proliferation of these lymphocytes [100].

Antisense against intercellular adhesion molecules

The binding of circulating leukocytes to vascular endothelium, which is mediated through intercellular adhesion molecules and their ligands, is an obligatory step in the emigration of leukocytes to the site of infection or injury. One such protein, ICAM-1, is expressed in both endothelial cells and non-endothelial cells, including leukocytes, fibroblasts, keratinocytes and other epithelial cells. Its expression can be induced by a number of cytokines including IL-1, TNFα and IFN-γ [99]. Antisense oligos that target human ICAM-1 mRNA inhibit the expression of ICAM-1 in human umbilical venous endothelial cells and human lung carcinoma cells [99]. Under equivalent experimental conditions treatment of endothelial cells with anti-ICAM-1 antisense blocked the adhesion of macrophages. Thus, the blockade of ICAM-1 expression was coincident with the loss of functional activity of the protein [99]. Recently, this antisense has been used in vivo and shown to be effective in delaying acute cardiac allograft rejection [94] and in inhibiting endotoxin-induced neutrophil emigration into the alveolar spaces [95].

Antisense against intracellular signal transduction proteins

Cytokine receptors are usually a multichain complex with ligand-binding chains that are specific for each cytokine and signal transducers that are common to several different cytokines [107]. Identification of cytokine-initiated specific signal transduction pathways may reveal clues for blocking cytokine-induced responses intracellularly. For example, a novel mitogen-activated protein kinase (MAPK), p38 MAPK, was recently identified from cells stimulated by lipopolysaccharide (LPS) [108]. Further studies have found that it may be activated in cells treated with cell wall components from Gram-positive bacteria, by pro-inflammatory cytokines such as TNFα and IL-1, by physical stress such as UV radiation or osmotic shock and by chemical stress such as H_2O_2 [109, 110]. p38 MAPK has been implicated in the phosphorylation of small heat shock proteins [110], activation of transcriptional factors [111] and in the expression of pro-inflammatory cytokines [109]. Thus, p38 MAPK activation may be essential for some cellular responses associated with acute or chronic inflammation [112]. Another member of the MAPK family, JNK, can also be stimulated by LPS in macrophages [113]. It is also designated as a stress-activated protein kinase, because of its activation upon stimulation by TNFα, UV radiation, and cellular stresses including heat shock and inhibitors of protein synthesis [114]. JNK has also been shown to be activated by ischemia-reperfusion in the kidney [115] and in the heart [116] and by acute elevation of blood pressure in rat arteries [117]. These novel protein kinases, with their upstream activators and downstream substrates form specific pathways involved in the cellular response to stress. Blocking these protein kinases with antisense oligos may be an effective approach to interrupt acute inflammatory reactions (Fig. 5).

Intracellular antibody targeting

More recently, it has been shown that synthesized single-chain antibodies can be transferred into a cell to target particular intracellular compartments, inhibiting virus replication at various stages. Modified antibodies can be stably expressed intracellularly within the nucleus and/or cytoplasm of eukaryotic cells and have been used to inactivate specific cellular gene products [118, 119]. The introduction of an antibody moiety into a cell that leads to the resistance of that cell to further infection by a viral pathogen has been entitled “intracellular immunization” [120]. This can be achieved by incorporating specific localization signals onto antibody chains or domains, conferring on them a new intracellular localization. Such fine control of the intracellular tar-

geting of antibodies has greatly increased the versatility of this immunological technique [121].

Human immunodeficiency virus type 1 (HIV-1) has a complex life cycle, which has made it a difficult target for conventional therapeutic modalities. A single-chain antibody moiety, directed against the HIV-1 regulatory protein Rev has been developed. This anti-Rev single-chain construct consists of both light and heavy chain variable regions of an anti-Rev monoclonal antibody which, when expressed in human cells, potentially inhibits HIV-1 replication. This intracellular SFv molecule has been demonstrated to be specific for antagonizing Rev function. Thus, intracelluar SFv expression, against a retroviral regulatory protein, may be useful as a gene therapeutic approach to combat HIV-1 infections [122].

Intracellular antibodies are likely to have widespread impact on biological research and as potential therapeutic agents [123]. Intracellular immunization has been succeessfully applied to inhibit the function of intracellular target proteins in a variety of different biological systems [121]. It is possible to use this technique to block the synthesis of pro-inflammatory cytokines or cytokine-induced intracellular signal transduction proteins (Fig. 1).

Molecular diagnosis

One of the proposed explanations for the lack of efficacy of anti-cytokine therapy is the difficulty in identifying the patients who might benefit from this type of therapy [124, 125]. It appears that there are no reliable clinical or physiological characteristics to help identify patients who could benefit from anti-cytokine therapy. One approach is to use an assay that can provide a rapid estimate of the particular cytokine, thereby identifying patients most likely to benefit. A rapid, semi-quantitative IL-6 dipstick test is currently being tested and may be useful in future clinical trials [8]. Another approach is to identify patients who may be particularly sensitive to the development of a disease or sensitive to poor outcome, based upon the expression of a gene dictating particular susceptibility. Thus, Stuber et al. [126] examined the allele frequency and genotype distribution of a biallelic TNF gene polymorphism in postoperative patients who had severe sepsis, using PCR to amplify a 782 base pair fragment of genomic DNA, which included the polymorphic site of the restriction enzyme NcoI within the TNF locus. They defined two alleles, TNFB1 and TNFB2, and found that non-survivors exhibited a significantly higher prevalence of the TNFB2 allele, with patients homozygous for this allele demonstrating a higher mortality rate than heterozygous patients ($p = 0.002$). Homozygous patients also had higher circulating TNF-α concentrations and higher multiple organ failure scores compared to heterozygotes. The pharmaco-genetic approach of using the underlying genetic make-up of a patient to ascertain prognosis and susceptibility to disease, as well as to direct therapy, is a very exciting one, which will probably increase in its use and utility in the coming years. In addition to its clinical utility, this approach will present complex ethical dilemmas with respect to the rationing of critical care services [127]. In the context of inflammatory cytokines, it is possible that particular patients may or may not respond to anti-cytokine therapy depending on the underlying genotype of the patient.

Bridging the gap

Newly developed anti-inflammatory approaches which are theoretically possible are summarized as examples to show the potential applications of molecular biotechnology in intensive care medicine. Currently, neutralizing antibodies and naturally expressed cytokine inhibitors have been subjected to clinical trials. Although gene therapy and antisense technology are still in their infancy, they are increasingly under examination in in vivo animal studies and limited clinical trials. Although none of these approaches have been shown to improve the clinical outcome of sepsis, ARDS and MODS, our understanding of the molecular mechanisms underlying these severe clinical conditions has been greatly advanced.

The ideal approach to be taken clinically depends on a greater understanding of the underlying biology (e. g., the inflammatory/anti-inflammatory cascade) as well as a better understanding of the patient's host defense system. Of interest is the difference in efficacy between interventions applied in many animal studies and the corresponding human investigations. Importantly, in many animal studies, therapeutic agents were given prior to the induction of sepsis, an approach that is not usually possible in patients. Secondly, these molecular-based approaches, with their inherent specificity, are usually designed to target a particular molecule. However, clinical disorders such as sepsis, ARDS and MODS, are complicated pathological processes. A therapeutic approach against one particular cytokine may not influence the complex cytokine network efficiently. The diversity of cytokine patterns may hold the key to the efficacy of the therapy. Therefore, advanced diagnostic methods to monitor patterns of cytokine activation in patients, and combined therapeutic methods may become the focus of future investigations. As mentioned above, humanized monoclonal antibody cocktails for a particular cytokine, or for several cytokines that have synergistic effect in the development of inflammatory reactions, could be more effective. By knowing the pattern of cytokines, a combination of different approaches should also be considered. Alternatively, blocking common intra-

cellular pathways initiated by multiple cytokines could also be more effective than approaches targeting only one cytokine. With our increased understanding at the molecular, cellular, physiological and clinical levels, the practice of molecular biotechnology on critically ill patients will hopefully soon be realized.

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