Getting to grips with the granulocyte: manipulation of granulocyte behaviour and apoptosis by protein transduction methods

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Abstract

Human granulocytes clearly play an important role in host defence against invading organisms, however they have also been implicated in the pathogenesis and progression of many chronic inflammatory diseases. In addition, these cells have been paramount in gaining a better understanding of many key-signalling pathways regulating fundamental biological processes. Since granulocytes are terminally differentiated and undergo relatively rapid constitutive apoptosis it has been difficult to manipulate intracellular events by transfection or transduction procedures. It has been shown in recent years that the HIV-TAT protein transduction system can be successfully used in granulocytes to manipulate key signalling mechanisms regulating functional responsiveness and survival. In this paper, we review recent literature highlighting important developments using this system in isolated human granulocytes and in inflammatory process in vivo where these cells play a prominent role.

The human polymorphonuclear neutrophil has been an invaluable and excellent cell for studies elucidating signal transduction mechanisms, especially signalling pathways involving phospholipid turnover (e.g. phosphatidylinositol metabolism, phospholipase C and D activity) and kinase systems (e.g. protein kinase C and PI3K (phosphoinositide 3-kinase)). The primary reasons for their use are that they are relatively easy to purify and high numbers can be readily obtained from blood of human volunteers. Neutrophils have obvious direct relevance to inflammatory processes, and findings obtained using these cells have clinical implications for the development of therapy for chronic inflammatory diseases, such as chronic obstructive pulmonary disease, rheumatoid arthritis, glomerulonephritis, myocardial reperfusion injury etc. However, there are some variables and factors that have to be considered. For example, variations in cellular responses are often observed using cells isolated from different donors and even from cells isolated at different times from the same donor. The culture conditions used can also determine the outcome of cellular responses. In particular, the density of neutrophils in culture can influence the rate of constitutive apoptosis [1]. The culture medium used is also an important factor that needs to be considered, e.g. cation (e.g. Ca²⁺ and Mg²⁺) concentrations, the presence of proteins (e.g. bovine albumin or serum versus human albumin or serum) and other constituents (e.g. Hepes, phenol red, nutrients etc.) may significantly affect cellular activity. The mode of cell isolation is also critically important since methods often require red cell lysis, changes of temperature during centrifugation steps, different reagents used during sedimentation (e.g. using gelatin, dextran etc.) and centrifugation (through gradients made with Percoll, Hypaque–Ficoll etc.) that can influence the biological activity of these cells. Recently, it has been shown that low-level contamination of other cells especially monocytes or eosinophils in neutrophil preparations may influence cellular function [2]. To remove contaminating cells, an extra procedure has to be introduced that uses antibody-dependent positive selection of the contaminating cells, a process that requires careful quality control assessment ensuring that neutrophil function remains unaffected. Another important issue that has to be addressed is the problem associated with protein degradation due to the high amounts of proteases inherently found in neutrophils and eosinophils. This has largely been remedied using techniques that use cocktails containing high concentrations of very powerful proteases [3,4]. Another consideration when working with granulocytes is the difficulty in transfecting these cells, since they are terminally differentiated and do not undergo cell division. However, it is now emerging that it is possible to manipulate key processes in granulocytes using efficient and rapid protein transduction techniques. It is crucial that any technique used to introduce proteins into granulocytes is fast since these cell die rapidly by apoptosis, a process that is underway within hours if not minutes once these cells have left the bone marrow. Moreover, it is critical that techniques used to introduce proteins into these cells do not interfere non-specifically with biological processes especially since cellular responsiveness of granulocytes can be easily influenced. In this paper, we have highlighted the attempts made by researchers to introduce proteins into granulocytes to manipulate key signalling mechanisms regulating functional responsiveness and survival.

Key words: apoptosis, eosinophil, granulocyte, HIV-TAT, neutrophil, protein transduction.

Abbreviations used: ERK, extracellular-signal-regulated kinase; LPL, lipoprotein lipase; NF-κB, nuclear factor κB; PI3K, phosphoinositide 3-kinase; PTD, protein transduction domain; Pyk2, proline-rich tyrosine kinase.

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For the transduction of proteins into granulocytes, the protein of interest is linked to positively charged PTDs (usually the 11-amino-acid HIV-TAT peptide). The protein enters the cell and/or nucleus via a predominately passive interaction with the negatively charged outer or nuclear membrane to exert biological effects.

There are several recent excellent reviews describing protein transduction technology in general that provide up to date information [5–9].

Unless a specific transporter system is active, all viable cells that have an intact cell membrane will exclude non-specific entry of macromolecules (>500 Da). There have been a number of techniques that have attempted to address this issue to introduce molecules into granulocytes to target specific intracellular targets. These techniques (including hypo-osmotic shock, electroporation, digitonin or saponin permeabilization and use of lipofectin) have had some limited success but in general it is likely that these procedures may non-specifically affect granulocyte function and therefore their usefulness is somewhat limited. More recently, it has been recognized that there are a relatively small number of proteins that are capable of entering directly into cells using as yet ill-defined mechanisms [5–9]. There are three well-established techniques and systems that have been successfully used to transduce proteins or peptides, compounds, nucleic acids and particles into both transformed and primary cells. All these techniques involve the use of small peptides or proteins that possess the ability to traverse the plasmalemma, or indeed nuclear membranes, using mechanisms that are generally believed to involve a passive mechanism that does not involve active transport, although there is evidence that the mode of entry may depend on the protein involved. Essentially, the three main systems involve the HIV-TAT (transactivator of transcription) protein transduction system, the Drosophila Antennapedia homoeotic transcription factor and the herpes simplex virus-1 DNA-binding protein, VP22. The protein segment responsible for actual cell entry has been termed the PTD (protein transduction domain). HIV-TAT technology has been the most successful mode of protein transduction in human granulocytes. The technique involves the synthesis of a fusion protein whereby the protein of interest is linked to the HIV-TAT transduction domain (a small peptide comprising the 11 amino acids YGRKKRRQRRR) using bacterial expression vectors. Procedures involve sonication of the bacterial pellet and denaturing the recombinant protein. The protein is then purified, usually involving Ni²⁺-nitrilotriacetate–agarose affinity column procedures, often followed by further purification and quality control steps. Cells in culture can then be treated directly with the purified protein. Protein transduction is concentration-dependent and highly efficient with reports of almost 100% of cells being transduced within 10 min. In addition, TAT-fusion proteins have been successfully used in experimental in vivo scenarios (see Figure 1).

Table 1 | The use of HIV-TAT-linked peptides in human granulocytes

<table>
<thead>
<tr>
<th>Granulocyte type</th>
<th>HIV-TAT linked peptide target</th>
<th>Primary functions investigated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil</td>
<td>LPL (an actin bundling protein)</td>
<td>Adhesion</td>
<td>[10]</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>β3-integrin cytoplasmic tail</td>
<td>Chemotaxis and transmigration</td>
<td>[11]</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>Pyk2</td>
<td>Spreading, respiratory burst, degranulation and killing of bacteria</td>
<td>[12]</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>NEMO (IKKγ) binding domain</td>
<td>Apoptosis and survival</td>
<td>[15]</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>RhoA (a Ras superfamily member of GTP-binding proteins)</td>
<td>Cell adhesion and detachment</td>
<td>[16]</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>H-Ras</td>
<td>Survival</td>
<td>[17]</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>Y-box-binding factor 1</td>
<td>Apoptosis and survival</td>
<td>[18]</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>Ras</td>
<td>Adhesion, airway inflammation and hyperresponsiveness</td>
<td>[19,21]</td>
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<tr>
<td>Eosinophil</td>
<td>PI3K</td>
<td>PLA2 activation, airway inflammation and hyperresponsiveness</td>
<td>[20,22]</td>
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TAT domain. These authors showed that these LPL-derived proteins rapidly (within 15 min) enter human leucocytes (neutrophils and monocytes) and induce leucocyte integrin-mediated adhesion. Interestingly, substitution of alanine for the serine abolished the ability of the peptide to induce adhesion. Thus by using such TAT-linked peptides, as well as other supportive evidence, these authors concluded that there is a novel role for LPL phosphorylation in the regulation of leucocyte integrin-mediated adhesion. The role of integrins in neutrophil responses was further developed by Bruyninckx et al. [11], who used HIV-TAT peptides corresponding to the β3-integrin cytoplasmic tail with modified tyrosine residues and identified the functional tyrosine residue responsible for its activation.

An important recent study by Han et al. [12], used a dominant-negative protein where the C-terminus of Pyk2 (proline-rich tyrosine kinase) fused to a TAT peptide but not other regions of Pyk2, inhibited specifically the respiratory burst of neutrophil responding to tumour necrosis factor, Salmonella or Listeria but not the response induced by the phorbol ester PMA. This study describes in detail the problems that can be encountered using this technology. They assessed uptake of fluorescently labelled TAT-tagged proteins using flow cytometric techniques and demonstrated that uptake depended on the method used to purify the peptides. For example, purification by ion exchange followed by gel permeation chromatography led to efficient and maximal uptake by 30 min. Importantly, other factors eluting from the column could induce toxic responses in the cells. However, if the peptides were desalted by membrane filtration they were not toxic. Uptake also increased with time over a period of 120 min at 37°C. Surprisingly perhaps, and unlike some other TAT peptides, there was no detectable uptake at 4°C. In addition, there was no saturation of uptake with concentrations of TAT peptides up to 1 μM and fluorescence was almost entirely intracellular. Interestingly, evidence was presented showing that lyososomal degradation can limit the accumulation and intracellular retention of the fluorescently tagged TAT protein. This study showed that optimal uptake required the presence of cations Ca²⁺ and Mg²⁺ and was insensitive to pH over the range 6.8–7.4. The authors chose peptides desalted by membrane filtration and a loading time of 30 min as this efficiently labelled the cells by approx. 90%. It was also noted that the endosomal compartment degraded the large amount of peptide that entered the neutrophils with only minute amounts reaching the cytosol. These authors attempted to introduce proteins using the Antennapedia system or a carrier peptide modelled on the sequence of the β3 integrin shown to enter myeloid cells directly. The authors were successful with only the HIV-TAT system and concluded that TAT-based transduction was the only approach that worked consistently in human neutrophils. Another important issue raised by Han et al. [12] is that, as mentioned above, the way the peptides are purified will also determine their relative toxicity and the fact that the peptides are prepared by bacterial expression systems means there is a realistic risk of introducing LPS (lipopolysaccharide) contamination. This latter point is particularly relevant in the investigation of neutrophil biology since LPS can stimulate human neutrophil responsiveness as well as influence rate of apoptosis and survival.

Recently, the HIV-TAT system has been successfully used in neutrophils and eosinophils to investigate signalling mechanisms regulating survival. We originally showed that NF-κB (nuclear factor κB) plays a critical role in human granulocyte apoptosis [13,14], since blockade of NF-κB activation by pharmacological inhibitors induced apoptosis in granulocytes and sensitized these cells to apoptosis induced by tumour necrosis factor α. These observations have recently been confirmed using the HIV-TAT protein transduction approach. Choi et al. [15] developed a specific inhibitor of NF-κB linked to a TAT-PTD to shuttle the inhibitor into human neutrophils. The peptide generated contained a NEMO (NFκB essential modulator)-binding domain that selectively inhibited IKKγ (NEMO)/IKKβ interaction to prevent agonist-induced NF-κB activation. Using this TAT peptide, constitutive apoptosis was accelerated and LPS-induced delay of apoptosis was attenuated. These authors carefully assessed the uptake of the TAT peptides using a combination of flow cytometry and confocal microscopy quality control checks. It was observed that almost 100% of the neutrophils were rapidly (within 10–20 min) stained with FITC-TAT, FITC-NEMO-binding domain and a FITC-control mutant peptide, with no non-specific effects of the TAT peptides. Interestingly, these authors reported (results not shown) that FITC-TAT peptides are effectively transduced into the entire neutrophil population in whole blood. This latter observation opens up the possibility of manipulation of intracellular pathways without the issues raised above concerning isolation procedures. To confirm our observation describing a role of NF-κB in eosinophil survival, we have recently generated a TAT peptide fused to a mutant of Itkβ (S32A and S36A) that is resistant to phosphorylation and proteasomal degradation. This TAT peptide efficiently blocked nuclear translocation of NF-κB in eosinophils and accelerated eosinophil apoptosis (S. Fujihara, R.T. Hay and A.G. Rossi, unpublished work).

There are now a number of publications demonstrating successful use of the HIV-TAT technology in eosinophils. The first publication to our knowledge to use a TAT peptide in eosinophils was by Alblas et al. [16]. These authors introduced, into human eosinophils, a TAT-fusion construct containing active RhoA, a member of the Ras superfamily of small GTP-binding proteins that controls actin stress fibre formation and focal adhesions. It was reported that in the presence of the chemotactrant platelet-activating factor the construct could stimulate detachment. Although the effect of the construct increased with time as concentrations of the peptide accumulated in the cells, the effect was lost after 45 min. The precise reason for this rapid loss of activity is unknown; however, it is possible that TAT peptides may be leaching out of the cell or that they are prone to proteolytic degradation. Interestingly, it was noted that in a small number of experiments the TAT construct had no effect. Although no explanation was presented, it is possible that the activation...
status of the eosinophils or donor variability may have been responsible.

It was subsequently shown that a TAT-linked dominant-negative H-Ras introduced into eosinophils inhibited ERK (extracellular-signal-regulated kinase) activation and interleukin-5-induced cell viability [17]. The TAT peptide entered eosinophils in a concentration- and time-dependent manner with >95% efficiency. Indeed, it was demonstrated that this TAT construct entered eosinophils within 20 min of treatment and effectively blocked the activation of ERK1 and ERK2 for at least 1 h. Interestingly, eosinophil viability prolonged by interleukin-5 was blocked by the construct even when measured after 60 h of eosinophil culture. In addition, another study investigated the mechanisms controlling GM-CSF (granulocyte/monocyte colony-stimulating factor) induced by effective use of the HIV-TAT technology [18]. A human Y-box-binding factor 1 linked to TAT, but not a control peptide (TAT-β-galactosidase), promoted eosinophil survival by stabilizing GM-CSF mRNA. Myuo and co-workers [19–22] have taken advantage of the HIV-TAT system to investigate a number of biological processes in eosinophils in vitro and inflammatory processes involving eosinophils in vivo. For example, they have used an HIV-TAT dominant-negative Ras to block focal clustering and active conformation in β2-integrin-mediated adhesion of eosinophils to intercellular cell-adhesion molecule 1 [19].

In addition, these authors have used an HIV-TAT-linked dominant-negative form of class 1A PI3K adapter subunit N to block fMLP (N-formylmethionyl-leucylphenylalanine)-stimulated phosphorylation of the downstream target of PI3K, protein kinase B, in human eosinophils [20]. Such effects helped to demonstrate that group IV cytosolic phospholipase A2 activation occurs by PI3K through a mitogen-activated protein kinase independent pathway [20]. Importantly, this group [21,22] has now shown that these HIV-TAT constructs can be used successfully in vivo to investigate inflammatory processes where these cells play an important role. Thus use of this technology will probably increase exponentially and undoubtedly will provide exciting advances in understanding granulocyte cell biology in vitro and inflammatory processes in vivo.

References


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