Differences in internalization and intracellular processing of interleukin-1 associated with the two forms of interleukin-1 receptor found in B-cells and T-cells

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There are at least two classes of interleukin-1 (IL-1) receptor, namely p80, an 80 kDa single-chain protein found in T-cells, fibroblasts and many other cell types, and p68, a 68 kDa protein expressed in B-cells and macrophages. The two classes of IL-1 receptor show distinct differences in their substrate-binding site and molecular properties. In this study we show that the kinetics of IL-1 internalization and the consequences of ligand processing in the two subtypes of IL-1 receptor are also very different. The Raji cell line was used as a source of the p68 form of the IL-1 receptor, whereas the YT cell line was used as a source of p80 receptor. Under conditions of steady-state binding the IL-1 was equally distributed between cell-surface and intracellular sites in YT cells, compared with predominantly cell-surface binding (85%) in Raji cells. The mechanism of IL-1 processing was also different in the two cell types. In Raji cells 60% of internalized IL-1 was released from the cells in an intact form, whereas the remainder was degraded. All of the IL-1 extruded from YT cells was intact. The kinetics of IL-1 release was faster in Raji cells, with a half-time of 4.5 h compared with over 15 h in YT cells. SDS/PAGE analysis of internalized IL-1 in Raji cells revealed that the ligand was sequentially processed to trichloroacetic acid-soluble products. The YT receptor–ligand complex was resistant to dissociation at pH 5, whereas that in Raji cells rapidly dissociated at this pH. Treatment of Raji cells with the lysosomotropic agent chloroquine inhibited the degradation of IL-1 without having any effect on the amount of intact IL-1 in the intracellular compartments. From these data we conclude that the pathways of internalization, intracellular trafficking and overall processing of IL-1 are different for p68 IL-1 receptors compared with p80. This could have direct consequences for IL-1 action and IL-1 receptor regulation in the cell.

INTRODUCTION

A variety of evidence from biochemical, immunological and molecular-biological studies suggest that there are at least two classes of interleukin-1 (IL-1) receptor, p80 and p68 [1–4]. T-cells, fibroblasts and endothelial cells express p80 [5], an 80 kDa single-chain protein whose identity has been established by molecular cloning [6]. By contrast, B-cells appear to express a 68 kDa IL-1 receptor, p68, with markedly different molecular properties from p80, including an ability to discriminate between IL-1 α and -1 β , and altered specificities for IL-1 analogues [1]. Since both molecules, p80 and p68, are differentially glycosylated to the same degree [7], the differences in molecular size must reflect changes in the polypeptide backbone of the IL-1 receptor.

Although there is considerable evidence supporting the idea of IL-1-receptor heterogeneity [1–7], the majority of this work has been aimed at delineating the molecular properties of the substrate-binding sites of the two proteins [1–3]. By contrast, there is a sparsity of information available regarding the intracellular fate of IL-1 and the IL-1 receptor [8–11]. A variety of isolated findings hint at differences in IL-1 and IL-1-receptor processing and intracellular routing [3,4,8–11]. For example, IL-1 appears to be degraded by B-cells [11], but not by T-cells [9], the internalized IL-1–receptor complex is extremely stable to dissociation in T-cells [9,12], and IL-1 has been shown to accumulate in the cell nucleus in both T-cells [9] and fibroblasts [10]. These observations hint at differences in IL-1 processing and intracellular routing in cells expressing the p68 IL-1 receptor compared with those expressing p80.

The present studies were initiated to define precisely the

mechanism of IL-1 processing in internalized IL-1–receptor complexes in B-cell and T-cell lines. In common with the considerable differences in functional and molecular properties of the two classes of IL-1 receptor described above, we show that the intracellular trafficking of IL-1 in internalized complexes of p80 and p68 IL-1 receptors is also markedly different. These findings could be of considerable importance in helping to understand the mechanisms that regulate IL-1 receptor signalling and the physiological response of the cell.

EXPERIMENTAL

Materials

Human recombinant IL-1 β expressed in *Escherichia coli* was purified to homogeneity by methods previously described [13]. ¹²⁵I-IL-1 (sp. radioactivity 143–180 μ Ci/ μ g), labelled with the Bolton–Hunter reagent, was from New England Nuclear. Reagents for electrophoresis were from Bio-Rad. Hepes and all other reagent-grade chemicals were from Sigma.

Cell culture

Raji cells were obtained from the American Type Culture Collection. YT cells were originally described by Yodoi *et al.* [14], and were maintained in RPMI 1640 medium containing 10% fetal-calf serum. The cells were passaged weekly and the medium was changed two additional times weekly. For binding assays the cells were collected, washed three times with RPMI 1640 and resuspended in binding buffer (RPMI 1640 containing 1% BSA and 20 mM-Hepes, pH 7.2). Cell viability was assessed

by Trypan Blue exclusion and by use of the redox dye MTT as described by Mossman [15]. Cell viability measured by these two assays was 90–95%. Cell number was determined with a haemocytometer.

Receptor-binding assay of ^{125}I -labelled IL-1 to intact cells

Raji and YT cells (1×10^7 cells/ml) were incubated with ^{125}I -labelled IL-1 in the presence and absence of unlabelled IL-1 at the appropriate temperature for the designated times. The incubation was terminated by removing samples from the cell suspension and separating cells from buffer by centrifugation through a silicone/paraffin-oil mixture as described previously [16]. Non-specific binding was determined in the presence of $1 \mu\text{M}$ unlabelled IL-1. The biological activity of the radiolabelled IL-1 in the mouse thymocyte proliferation assay [17] was the same as that of unlabelled IL-1 (results not shown).

Acid extraction procedure

The distribution of ^{125}I -labelled IL-1 between the cell surface and the cell interior was determined by quantifying the total amount of IL-1-associated binding as described above and then subtracting from this the amount of labelled ligand associated with the cell interior to yield an estimate of surface-bound ligand. Cell-associated radioactivity was determined by acid extraction of cells which removed cell-surface-bound material [18]. Acid wash of the cells had no effect on their viability measured by the MTT assay.

Determination of intact and degraded IL-1

Degradation products of IL-1 in solubilized cell extracts or in the extracellular medium were assessed by determining the trichloroacetic acid solubility of ^{125}I -IL-1 products. Radioactive material precipitable in 10% trichloroacetic acid was considered intact IL-1. In addition, the molecular size of the radiolabelled IL-1 products was confirmed by SDS/PAGE.

Treatment of cells with chloroquine

Cells were incubated with ^{125}I -IL-1 in the absence and presence of $100 \mu\text{M}$ -chloroquine at 37°C for 8 h. Samples were removed at the times indicated, cooled to 4°C , acid-stripped as described above to dissociate surface-bound ligand and then solubilized with 10 mM-CHAPS in the presence of a cocktail of protease inhibitors including leupeptin, aprotinin and pepstatin (each 0.5 mg/ml) and 0.2 mM -phenylmethanesulphonyl fluoride. The solubilized cells were then precipitated with trichloroacetic acid to assess the amount of intact IL-1. In some experiments trichloroacetic acid-precipitated samples were solubilized with SDS and analysed by SDS/PAGE to determine the composition of the internalized radioligand.

SDS/PAGE

Acid-stripped Raji and YT cells (5×10^6 cells) were centrifuged ($10000 g_{\text{max}}$ for 10 min) and the cell pellets solubilized by boiling for 3 min in sample buffer (1% SDS/50 mM-dithiothreitol). The solubilized cells were then analysed by SDS/PAGE on 1.5 mm-thick slab gels. Resolving gels of 15% acrylamide were used, and electrophoresis was carried out at 30 mA per gel. Prestained ^{14}C -labelled molecular-mass marker proteins (Amersham) were used to estimate the molecular mass of the radiolabelled membrane proteins.

Dissociation experiments

Cells were incubated with ^{125}I -IL-1 for 6 h at 4°C in pH 7.6 binding buffer. The cells were then washed rapidly to remove unbound IL-1 and then re-incubated in the original volume of binding buffer adjusted to pH 7.6 or pH 5.0. Samples were

removed at the times indicated and centrifuged through oil to separate free from bound IL-1 as described above.

RESULTS

The time course at 37°C of IL-1 binding and internalization to cell-surface receptors is shown in Fig. 1 for Raji and YT cells. Binding to cell-surface IL-1 receptors was around 15 times faster in YT cells than in Raji cells ($t_{1/2}$ of association 5 min and 60 min respectively). In addition the internalization rates differed between the two cell types. Kinetic transformation of steady-state IL-1-binding data (measured after 4 h of incubation; Fig. 1) indicates that IL-1-receptor internalization is around 25 times faster in YT cells than in Raji cells. This is reflected in the steady-state distribution of IL-1 receptors, which in YT cells is almost equally distributed between surface and intracellular sites, in contrast with Raji cells, where only around 16% of binding is intracellular under such conditions (Fig. 1). Whether these

Fig. 1. Steady-state binding of ^{125}I -IL-1 in Raji and YT cells at 37°C

Curves shown represent cell-surface-associated IL-1 β (○) and internalized IL-1 β (●). Cells were incubated with ^{125}I -IL-1 β , and samples were removed and centrifuged through oil at the times indicated. Internalized ^{125}I -IL-1 β was distinguished from surface-bound by a 3 min wash with glycine/HCl (pH 3.0). Non-specific binding was less than 10% of total binding for both Raji and YT cells. Data shown are representative from four separate studies.

Fig. 2. Release of total internalized ^{125}I -IL-1 in Raji and YT cells at 37°C

Cells were preloaded with ^{125}I -IL-1 β for 2 h at 37°C . After washing in pH 3.0 buffer to remove extracellular and surface-bound IL-1, the cells were re-warmed to 37°C , and samples were removed and centrifuged through oil at the times indicated. The released ^{125}I -IL-1 represents total ligand radioactivity (c.p.m.). Data shown are representative from four separate studies.

Fig. 3. Release of intact and degraded internalized ^{125}I -IL-1 in Raji and YT cells at 37°C

Curves shown represent (○) intact IL-1 and (●) degraded IL-1. Experimental conditions were as in the legend of Fig. 2, except that the released radioactivity was assessed as intact or degraded by precipitation with trichloroacetic acid and by SDS/PAGE analysis. Data shown are representative from four separate studies.

differences reflect inherent properties of the two types of IL-1 receptor or whether they reflect differences in cellular architecture between Raji and YT cell lines in unknown. Nevertheless, in the light of these observations we chose to determine the fate of the internalized receptor complexes in these two cell types to see whether differences in IL-1 and IL-1-receptor processing could also be observed.

To determine the rate at which internalized radiolabelled IL-1 was released from Raji and YT cells, ^{125}I -IL-1 was preloaded into the cell interior at 37°C , the cells were cooled to 4°C , all surface-bound IL-1 was removed with a pH 3 wash, and then the cells were re-warmed to 37°C for the times indicated. As shown in Fig. 2, the release of IL-1 from Raji cells was curvilinear with time, consistent with complex kinetics indicative of a multi-exponential system. The half-time of IL-1 extrusion varied from around 4.5 to 5.5 h. In contrast with Raji cells, the extrusion of IL-1 from YT cells was a much slower process, with a $t_{1/2}$ of

around 15–16 h (Fig. 2). Because of the length of some of the incubations (12–16 h), we were concerned that the differences in IL-1 release between the two cell types might reflect differences in cell viability. To eliminate this possibility, cell samples were tested for viability at each time point; this, based on the MTT assay [16], was 90–95%.

In order to understand the differences in the kinetics of IL-1 release in these two cell types, we measured the relative proportions of intact versus degraded IL-1 that was extruded with time. As shown in Fig. 3, Raji cells extruded both intact and degraded IL-1 into the extracellular medium. Analysis by SDS/PAGE of the IL-1 extruded from the cells revealed substantial radioactivity running at the dye front (results not shown), presumably representing small labelled peptides. The kinetics of release of intact versus degraded IL-1 differed in several ways. First, the release of intact IL-1 was quite rapid, with a $t_{1/2}$ of 30 min (Fig. 3). In contrast, the release of degraded IL-1 was a much slower process (Fig. 3). Second, the release of intact IL-1 reached a plateau after around 3 h, while the release of degraded IL-1 was still increasing linearly with time. In contrast with the findings in Raji cells, the IL-1 released from YT cells was almost entirely intact (Fig. 3). In some experiments degradation of IL-1 in YT cells was observed; however, this was always less than 2% of total released IL-1. Thus the kinetic differences in the rate at which intact and degraded IL-1 are released from Raji cells are consistent with the presence of two separate pathways of IL-1 processing in these cells: (1) a rapid retroendocytotic pathway of release for the intact IL-1, and (2) a slower degradative pathway of IL-1 processing.

The processing of internalized ^{125}I -IL-1 in these two cell types was further examined by SDS/PAGE analysis (Fig. 4). Cells were incubated at the time points indicated, samples were removed, cooled to 4°C , and extracellular and surface-bound IL-1 were removed with a pH 3 wash. After this procedure, the acid-stripped cell pellets were solubilized in SDS sample buffer and subjected to SDS/PAGE. In Raji cells there was a time-dependent increase in accumulation of intact 18 kDa ^{125}I -IL-1 (Fig. 4). In addition there was also a time-dependent increase in radioactive proteins of molecular masses 12 kDa and 8 kDa, presumably reflecting degradation products of IL-1. After 8 h the degraded IL-1 represented around 30% of the total radioactivity on the gel. A similar time course of processing was seen in two other cell lines, WIL-2 and RPMI 1788 (results not

Fig. 4. SDS/PAGE analysis of internalized ^{125}I -IL-1 in (a) Raji cells and (b) YT cells at 37°C

Cells were incubated with ^{125}I -IL-1 β at 37°C ; at the times indicated samples were removed and washed with pH 3.0 buffer to strip away surface-bound IL-1. Cellular proteins including internalized ^{125}I -IL-1 were solubilized in SDS sample buffer analysed on SDS/15%-acrylamide gels. Positions of molecular-mass markers are shown. Data shown are representative from five separate studies.

Fig. 5. Dissociation of surface-bound ^{125}I -IL-1 from Raji ($\square$, $\blacksquare$) and YT ($\circ$, $\bullet$) cells as a function of pH at 4 °C

Cells were incubated with ^{125}I -U-IL-1 β for 6 h at 4 °C. The cells were then washed rapidly to remove unbound IL-1 and then re-incubated in the original volume of pH 7.6 ($\square$, $\circ$) or pH 5.0 ($\blacksquare$, $\bullet$) binding buffer. Samples were removed at the times indicated and centrifuged through oil to separate free IL-1 from bound. The bound radioactivity represents intact IL-1 as assessed by trichloroacetic acid precipitation of the dissociated IL-1. Data shown are representative from four separate studies.

shown), which express the p68 form of the IL-1 receptor (J. G. Giri, G. Sandlin, R. C. Newton & R. Horuk, unpublished work). In contrast with these findings, the SDS/PAGE analysis of internalized ^{125}I -IL-1 in YT cells revealed a time-dependent increase of intact IL-1, with no evidence of IL-1 degradation (Fig. 4b). These data are consistent with those showing the absence of extruded IL-1 degradation products from YT cells (Fig. 3).

To provide a molecular explanation for the observed differences in the processing of internalized IL-1 between Raji and YT cells, we examined the stability of IL-1/IL-1-receptor complexes under conditions resembling those found in endosomes, i.e. dissociation at pH 5. Raji and YT cells were incubated with radiolabelled IL-1 at 4 °C for 6 h. At 4 °C there is no internalization of IL-1, and binding is solely to cell-surface receptors [1]. The cells were washed to remove unbound ligand and then resuspended in the original volume of either pH 7.6 or pH 5 binding buffer. The dissociation of surface IL-1 with time was monitored at both pH values, and the data are shown in Fig. 5. The dissociation of IL-1 from Raji- and YT-cell IL-1 receptors at pH 7.6 was extremely slow; after 2 h only around 10% had dissociated. Interestingly, the dissociation of IL-1 from YT cells

was also resistant to incubation in the pH 5 binding buffer (after 2 h only around 12% had dissociated). By contrast, receptor-bound IL-1 in Raji cells was very susceptible to dissociation at pH 5; after only 2.5 min, 35% had dissociated, and at 2 h dissociation was almost complete. Other cells that express the p68 IL-1 receptor (WIL-2 and RPMI 1788 cells) showed a similar pattern of dissociation to that observed in Raji cells (results not shown).

If IL-1 goes through a degradative pathway of processing, as we have suggested in Raji cells, then the addition of agents such as chloroquine, which have been shown to inhibit lysosome-dependent hormone degradation [19,20], should lead to an accumulation of intracellular IL-1. To test this idea, we incubated Raji cells with labelled IL-1 in the presence and absence of chloroquine at 37 °C for 8 h. At selected times, samples of the cells were removed and acid-stripped to remove cell-surface-bound ligand and then solubilized in CHAPS to release the internalized IL-1. The latter material was precipitated with trichloroacetic acid to separate intact from degraded ligand (Fig. 6). In control Raji cells the ratio of intact to degraded IL-1 was around 3:1 after 8 h, consistent with data presented above (Fig. 3). Treatment of the cells with chloroquine dramatically altered this ratio to 10:1 in favour of intact versus degraded IL-1 (8 h time point). In separate experiments we found that chloroquine had no effect on the internalization rates or the amount of IL-1 bound to cell-surface receptors (results not shown).

DISCUSSION

After binding and internalization, the IL-1-receptor-ligand complex is sorted and targeted toward a processing pathway which decides its intracellular fate. The nature of the sorting process for these complexes is unknown. After internalization, around 60% of ligand is released from B-cells in an intact form by retroendocytosis [21–23]. This process is not affected by treatment of cells with chloroquine (Fig. 6). In T-cells the release of intact IL-1 is slower (Fig. 3) and comprises only 25–30% of total internalized IL-1.

Although not formally shown, it is assumed that after retroendocytosis the remainder of the receptor complex enters an endosomal compartment. The p68 IL-1 receptor complex dissociates fairly rapidly in the acidic environment of the endosome, and after fusion with lysosomes around 40% of total internalized IL-1 is degraded by lysosomal enzymes. The degradative pathway is inhibited by chloroquine (Fig. 6), and the slower kinetics of release of the degraded IL-1 (Fig. 3) are consistent with passage through lysosomal compartments. The fate of IL-1 in the Raji cells thus mirrors that of such well-characterized ligand-receptor systems as low-density lipoprotein, asialoglycoprotein and insulin [22–24]. As with IL-1 bound to the p68 receptor, the latter receptor-ligand complexes dissociated in endosomes, and the free ligand was either degraded in a chloroquine-sensitive lysosomal-dependent manner or extruded from the cell in an intact form via a retroendocytotic pathway [22–24]. By contrast, the p80 IL-1 receptor complex is resistant to dissociation in the endosome, and thus follows a different fate. Unlike the human chorionic gonadotropin receptor complex, which is also resistant to endosomal dissociation and is degraded in lysosomes [25], the IL-1 internalized by p80 receptor undergoes little degradation of ligand.

On the basis of the data discussed above, the differences in IL-1 processing observed in the two different cell types probably reflect differences in IL-1 binding to the two types of IL-1 receptor. The sensitivity of the p68 IL-1 receptor complex, but not the p80 IL-1 receptor complex, to acid dissociation (Fig. 5), for example, is likely to manifest itself as differences in stability

Fig. 6. Effect of chloroquine on the time course of accumulation of intracellular ^{125}I -IL-1 in Raji cells

Cells were incubated with ^{125}I -IL-1 in the absence ($\bullet$, $\circ$) or presence ($\blacksquare$, $\square$) of 100 μM -chloroquine at 37 °C. Samples were removed at the times indicated, cooled to 4 °C, acid-stripped to dissociate surface-bound ligand and then solubilized with 10 mM-CHAPS. The solubilized cells were then precipitated with trichloroacetic acid to assess the amount of intact ($\circ$, $\square$) and degraded ($\bullet$, $\blacksquare$) IL-1. Data shown are representative from two separate studies.

in endosomal compartments and may account for the different fates of the internalized IL-1 in the two cell types. An alternative explanation for the data is that the two cell types process IL-1 differently, not because their receptors are different, but because of other differences in their intracellular machinery, i.e. endosomes for protein sorting and handling. This is unlikely, since it is known that YT cells can bind and internalize IL-2, and the IL-2-receptor-ligand complex is sensitive to dissociation at pH 5 [26]; R. Horuk, unpublished work). In addition, a similar resistance to dissociation for IL-1 bound to the p80 receptor was observed in pig synovial cells [27].

Endocytosis of IL-1 receptor complexes may play a role in IL-1 signal transduction in some cell types. In line with this idea, variants of the murine EL4 cell line that failed to internalize IL-1 also failed to induce interleukin-2 receptor in response to IL-1 stimulation [28]. Although the nature of the signal is unknown, it could be provided by either internalized IL-1 or IL-1 receptor. However, the role of IL-1-receptor-mediated endocytosis in mediating biological effects of IL-1 is still controversial and remains to be elucidated. Alternatively, the internalization and degradation of IL-1 and the IL-1 receptor might serve as a mechanism to switch off IL-1 signals, desensitizing the cell to its ligand. The differences in IL-1 processing observed here could thus be of physiological significance either in IL-1 signal transduction or in serving to terminate IL-1-mediated signalling processes.

It is clear from studies with other hormone receptors [29] that the intracellular sorting and processing of the receptor and the internalized ligand are under the control of the cytoplasmic domains of the respective receptors. It is possible that differences in the cytoplasmic domains of p68 and p80 could account for the different patterns of IL-1 processing and intracellular handling observed here. In support of this idea are the recent findings demonstrating that the p68 and p80 IL-1 receptors are separate gene products [3,4]. From these findings it is likely that there are considerable differences in primary amino acid sequence of the extracellular and intracellular domains of the two classes of IL-1 receptor. Thus, by analogy with other polypeptide hormone receptors [29–35], the differences in the intracellular handling of IL-1 discussed above could reside in potential differences in the cytoplasmic domains of the p68 and p80 IL-1 receptors.

In conclusion, the present studies suggest that there are separate pathways of IL-1 processing in cells expressing the p68 IL-1 receptor compared with those expressing p80. The fate of internalized IL-1 and IL-1 receptor and the intracellular pathways that they traverse could reflect differences in the molecular properties of p68 and p80, and may play a role, directly or indirectly, in signal transduction. Thus elucidation of the pathways of IL-1 and IL-1-receptor processing could provide insight into the mechanisms that regulate IL-1-receptor signalling and may be of direct physiological importance in the cell.

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