Original Article

Isolation of Granulocytes: Which Transcriptome Do We Analyse – Neutrophils or Eosinophils?

(total RNA / gradient centrifugation / cell isolation / magnetic separation / gene expression / expression activity / neutrophils / eosinophils / granulocytes)

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Abstract. Isolation of granulocytes from blood is necessary for accurate study of changes in their expression. After gradient centrifugation, we obtain relatively pure granulocyte populations with different ratios of neutrophils and eosinophils. Unfortunately, in many studies in this field the expression results are not set according to the real variability of the granulocyte population. In many cases, the granulocyte population is marked simply as "neutrophils" and the residual population of eosinophils is not considered. Based on our recent study where we tracked the general transcription factor RNA polymerase II, we hypothesized that eosinophils are more transcriptionally active cells than neutrophils. We decided to test our hypothesis on isolated cells because its implications could change our view on many past expression analyses performed on granulocytes. In our experiments, we isolated neutrophils and eosinophils and measured their total RNA production. According to our results, eosinophils produce much more RNA than neutrophils. Therefore, relatively low numbers of highly active eosinophils can markedly affect the whole pool of granulocytic RNA. We want to emphasize that either a detailed description of the cell population or the use of a pure neutrophil population is necessary for the correct interpretation of neutrophil expression analysis results.

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Abbreviations: EDTA – ethylenediaminetetraacetic acid, FACS – immunofluorescent separation, LPS – lipopolysaccharide, MACS – immunomagnetic separation, RNAP II – RNA polymerase II.

Introduction

Various granulocytes are similar in shape, but not so in their different functions. Neutrophils, eosinophils and basophils are distinguished by the presence of cytoplasmic granules. These cells have shrunken and lobulated nuclei with highly condensed chromatin, but they differ in the number of lobes. Neutrophils, the most common type of granulocytes in human blood, contain nuclei composed of three to five lobes. Neutrophils are the first defenders against bacterial, viral, fungal and multicellular pathogens and are the cornerstone of congenital immunity. They also respond to many signals and produce a variety of molecules that play a role in inflammation, tumour immunity, transplant rejection, and autoimmune syndromes. Eosinophils also protect the body against pathogens and parasites. Their granules are packed with huge amounts of RNases that help fight against viral infections (Domachowski and Rosenberg, 2000). Along with basophils, they control the processes associated with allergies and asthma.

To study changes in the function and expression in granulocytes, the purest cell population is needed. There are different ways to isolate granulocytes. The most common way is based on the different densities of mononuclear cells, basophils (< 1.077 g/ml), and other granulocytes (> 1.077). The blood is layered on non-toxic liquid with a density of 1.077 g/ml, e.g., Percoll™, Histopaque™. After centrifugation, the cells with a higher density have settled at the bottom and can be easily separated. The method is relatively inexpensive and we can obtain pure (> 95%) and viable (> 95%) cell populations of granulocytes. A much more effective method is based on antibodies against specific markers on the surface of granulocytes. These immunomagnetic (MACS) or immunofluorescent (FACS) separation methods guarantee a very high purity (> 98%) of isolated cell populations of neutrophils or eosinophils.

Along with purity, it is necessary to keep an eye on unwanted cell activation. Activation can dramatically change viability, responsiveness, and overall gene expression of cells. It was described that after gradient centrifugation, neutrophils show significantly higher ac-

tivation compared to MACS (Zahler, 1997). The level of activation can be lowered by adding ethylenediaminetetraacetic acid (EDTA) as an anticoagulant after blood collection (Freitas et al., 2008), working with cells at a lower temperature after isolation, or using washing buffers without Ca^{2+} and Mg^{2+}.

When isolating eosinophils, the issues of cell activation are different. Eosinophils are often purified by negative selection from granulocytes or from whole leukocytes. CD16 is a specific marker of neutrophils. In a $CD16^+$ cell depletion kit (Miltenyi Biotec, Bergisch Glandbach, Germany), eosinophils become activated, and their chemotactic migration is affected (Rozell et al., 1996; Sedgwick et al., 1996). Another isolation kit with lineage depletion (CD2, CD14, CD16, CD19, CD56, CD123, CD235a cell depletion kit; Miltenyi Biotec) shows even worse results. This isolation kit also affects cell viability, increasing the apoptosis ratio and cell activation. Some authors do not recommend this kit for functional investigation of eosinophils (Schefzyk et al., 2009). Another, very elegant method for eosinophil isolation uses hypotonic lysis. This method activates only a small number of cells. However, hypotonic lysis is usable only for blood containing a high proportion of eosinophils (Samoszuk, 2006).

Based on the literature, MACS separation is probably the best method for neutrophil-focused experiments. Even if the eosinophils are isolated by negative MACS selection, the problem with their activation remains.

After gradient centrifugation, each sample contains neutrophils, a subpopulation of eosinophils, and a small number of monocytes. However, investigators look at such populations differently. Some consider them just granulocytes, while some ignore the eosinophil and monocyte subpopulations and simply call it a neutrophil population. Such studies ignore the cell population variability because the number of eosinophils is so low compared to neutrophils. Other scientists account for the variability, but with the presumption that eosinophil expression is very similar to neutrophil expression.

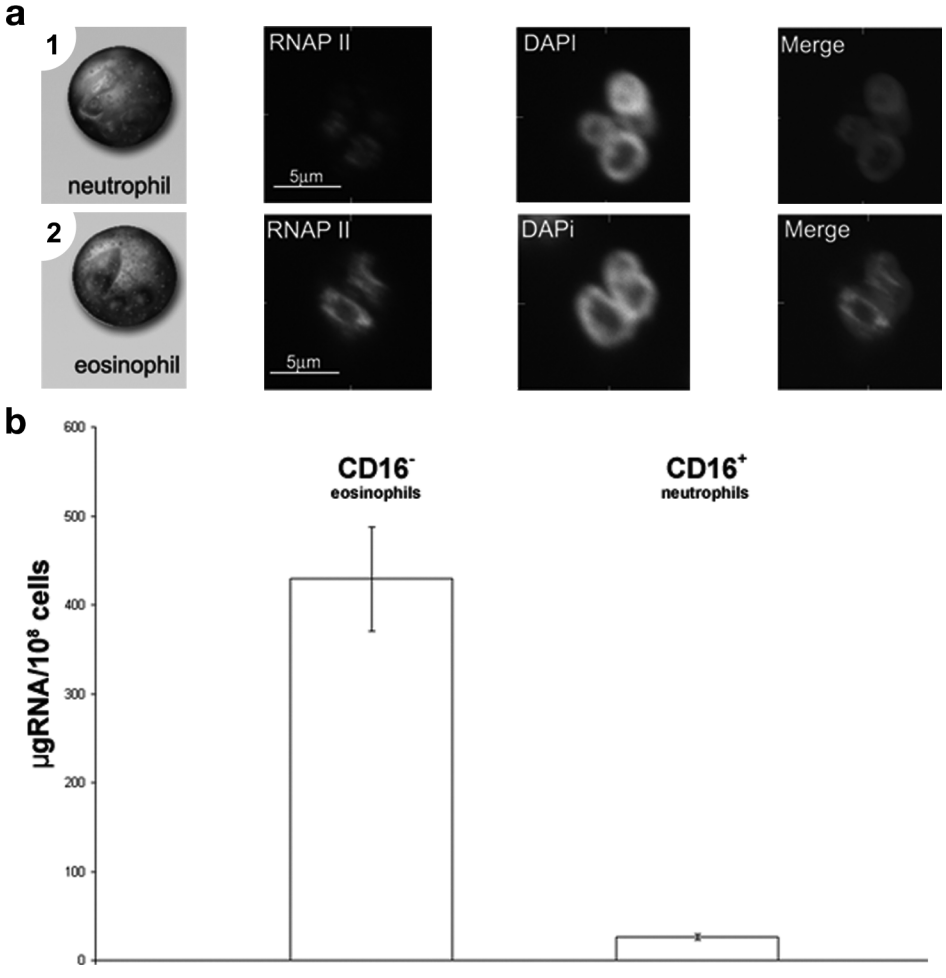

Fig 1. Different overall expression levels in granulocyte and eosinophil cell population
a. RNAP II distribution in (1) eosinophils and (2) neutrophils during the terminal differentiation stages of granulocyte development. Slices of cells obtained by confocal fluorescent microscopy. (RNAP II) FITC-labelled RNA polymerase II, (DAPI) DAPI-labelled chromatin, (Merge) merged image. DAPI staining shows highly condensed chromatin that is located predominantly in the lobes' periphery. The highly homogenous foci of RNAP II are located in the inner part of the eosinophils' lobes. Conversely, neutrophils contain very small amounts of RNAP II in their nuclei.
b. Differential amount of RNA in mg/10^8 cells in the eosinophil ($CD16^-$) and neutrophil ($CD16^+$) cell populations

In our previous experiments, we studied the distribution of a major transcription factor, RNA polymerase II (RNAP II) (Stejskal et al., 2010). We found that neutrophils contain a negligible amount of RNAP II in contrast to eosinophils (Fig. 1a). This is very interesting since we expected the same level of transcription in these two types of granulocytes. If RNA production depends on the RNAP II level, why do the cells with the high amount of RNAP II produce a similar amount of RNA as cells with less RNAP II? Since no such information was available, we hypothesized that a different path exists for the overall regulation of RNA. Another theory says that the RNAses from eosinophils lowered the amount of RNA during isolation (Hämäläinen et al., 1999). In this letter, we inspected the real amount of total RNA in observed cell types. At first, granulocytes were isolated by density centrifugation, then the CD16-positive fraction of neutrophils and CD16-negative fraction of eosinophils were separated. In the end, the amounts of RNA per 10^8 cells were calculated.

Material and Methods

Human heparinized peripheral blood was obtained from healthy donors in concordance with the Helsinki guidelines. Human granulocytes were isolated using the Histopaque-1077 and Histopaque-1119 gradient density centrifugation (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Residual erythrocytes were removed by washing with an erythrocyte lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, and 2mM EDTA) for 15 min at 4 °C. Monocytes were removed by cell incubation in culture flasks with RPMI medium (Invitrogen, San Diego, CA) for 30 min at 37 °C. After that, neutrophils were separated from granulocytes by immunomagnetic separation with CD16 MicroBeads (Miltenyi Biotec) according to the manufacturer's protocol. The cells were counted in a Bürker counting chamber and the results were confirmed in a Countess™ Cell Counter (Invitrogen). Purity of cells was established with a Zeiss S100 fluorescent microscope (Carl Zeiss MicroImaging, Göttingen, Germany). We took advantage of DAPI staining of nuclei and non-specific autofluorescence for eosinophils, neutrophils, and mononuclear cell identification (Weil and Chused, 1981). RNA from $CD16^+$ and $CD16^-$ cells was isolated with the TRI reagent (Sigma-Aldrich) according to the manufacturer's protocol. The quantity and purity of the isolated RNA were measured with a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). The RNA content in granulocytes was measured in μg per 10^8 cells.

Results and Discussion

After MACS separation, we obtained pure $CD16^+$ neutrophil (~98%) and $CD16^-$ eosinophil populations (purity about 95%) (Table 1). In contrast with the general concept, the RNA expression in $CD16^-$ cells was significantly different than in neutrophils ($P = 0.006$, Mann-Whitney test). Furthermore, an average total amount of RNA per 10^8 cells was 16.43 times higher in the $CD16^-$ eosinophil population than in neutrophils (Fig. 1b).

What do these results mean in practice? We have donor blood that contains 50 % neutrophils, 2.5 % eosinophils and 47.5 % mononuclear cells. After mononuclear cell depletion via gradient centrifugation, we obtain a granulocyte population. In an ideal case, these granulocytes consist of ~5 % eosinophils and 95 % neutrophils. At this point, the eosinophils form the minority in the whole granulocyte population. Nevertheless, the high expression activity of these cells is obvious after RNA isolation, when about 46 % of the total RNA from the granulocyte sample belongs to the eosinophils. From our observations, the granulocyte population was composed of 2–8 % eosinophils after gradient centrifugation and 30–63 % eosinophil RNA. With a wide variance of eosinophil content in the blood (-5 % of leukocytes), the neutrophil RNA may be contaminated with 0 to 67 % of eosinophil RNA after RNA isolation. This finding demonstrates the necessity of describing a granulocyte population before the results are interpreted.

This result shows that the $CD16^-$ fraction with 95 % eosinophils contains more transcriptionally active cells than neutrophils. Higher expression activity corresponds with the RNAP II distribution in their nuclei. Whereas the neutrophil nuclei contain negligible amounts of RNAP II, eosinophil nuclei show a bright signal comparable to protein concentration. A recent study compared the functional and expression differences between pure populations of eosinophils and neutrophils. Eosinophils express genes related to DNA repair that are necessary for dealing with double- and single-strand DNA breaks. Eosinophils also exhibit nucleolar activity. Neutrophils lack both of these abilities (Salati et al., 2007). Functional activity can be affected by residual cells in a granulocyte population. $CD14^+$ monocytes influence the li-

Table 1. Leukocyte differential count during granulocyte isolation

The distribution of neutrophils, eosinophils and cells without segmented nuclei (monocytes, lymphocytes) after different steps of granulocyte isolation (median ± SE, N = 10). Differential count of cells was established with a Zeiss S100 fluorescent microscope after DAPI staining.

	Neutrophils (%)	Eosinophils (%)	Mononuclear cells (%)
After Histopaque (1077/1119) isolation	90.6 ± 2.5	4.8 ± 2.0	4.6 ± 1.7
MACS CD 16^+ isolation	97.3 ± 1.4	0.4 ± 0.2	2.4 ± 1.5
MACS $CD16^-$ isolation	2.6 ± 1.8	95.2 ± 2.5	2.2 ± 1.5

popolysaccharide (LPS)-based activation of neutrophils. Moreover, granulocytes without monocytes do not show prolongation of lifespan after treatment with LPS (Sabroe et al., 2002, 2004).

The overall expression level of $CD16^+$ neutrophils is significantly lower than the expression of $CD16^-$ eosinophils. Neutrophils are cells with very low gene expression levels, and even a residual amount of highly transcriptionally active eosinophils can dramatically change the view of the neutrophil gene expression. When gradient centrifugation for granulocyte isolation is used, one must keep in mind that the resulting population consists of a set of two subpopulations with very different RNA content and a different gene expression profile. We calculated different RNA ratio of granulocytes after their isolation from peripheral blood of healthy volunteers. In case of bone marrow or blood from patients with myeloproliferative disorders, the $CD16^-$ fraction is also enriched by immature cells from granulocyte development (Cowland and Borregaard, 1999). Granulocyte populations should be well described in the articles dealing with gene expression experiments (RT-PCR, microarrays, etc.) or even better, pure neutrophil or eosinophil populations should be used.

The authors declare that they have no conflict of interest.

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