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Chapter 6

Phenotypic characterization of human intraplaque mast cells using flow cytometry

Manuscript in preparation

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Abstract

Human mast cells have previously been associated with adverse cardiovascular events. Mast cell activation, through the classical antigen sensitized-IgE binding to their characteristic Fcε-receptor, causes the release of their cytoplasmic granules. These granules are filled with neutral proteases, such as chymase and tryptase, histamine and pro-inflammatory mediators. Mast cells accumulate at high numbers within the human atherosclerotic tissue, particularly in the shoulder region of the plaque. However, their activation mechanisms, as well as their protease content, locally in the plaque, are still largely unclear. This is the first study to use flow cytometry for the analysis of the mast cell content in 22 human plaque samples, collected after femoral and carotid endarterectomy surgery. We observed that most intraplaque mast cells are activated, based on their CD63 protein expression. Furthermore, we detected that most of the activated mast cells had IgE fragments bound on their surface, while a fraction showed IgE-independent activation. Finally, we confirm previous reports stating that, while the majority of mast cells contain both chymase and tryptase, there is remarkable heterogeneity in the distribution of these proteases within the mast cell population per individual. In conclusion, this project establishes the strong relation between the presence of IgE and the activation of mast cells, which leads to the subsequent secretion of their protease content inside atherosclerotic plaques. Our data pave the way for potential therapeutic intervention through targeting IgE-mediated actions in human atherosclerosis.

Report

Up to the present day, atherosclerosis, the main underlying pathology of acute cardiovascular syndromes like stroke or myocardial infarction, is the major cause of human mortality[1]. As in most pathological conditions, the response of the immune system is crucial in the advancement of atherosclerosis, with the mast cells being key mediators in this process[2]. Mast cells are innate immune cells, mainly characterized by their notorious granular load release, upon activation with antigen-sensitized IgE fragments in allergy[3]. Aside from allergic inflammation, mast cells have long been established to play an important pro-inflammatory role in the development of atherosclerosis, as detected by both experimental studies as well as in human subjects[4]. Mast cells reside at low numbers in normal arterial tissue, however they are found to accumulate in arteries where a lipid-rich atherosclerotic plaque is formed[5]. In fact, as human atherosclerosis progresses, mast cells become increasingly activated whereupon they excrete their granules in the surrounding tissue[6]. The degranulated material consists mainly of proinflammatory cytokines, histamine and neutral proteases, such as chymase and tryptase[7]. Mast cell activation is reported to augment plaque progression[8], enhance plaque destabilization[9] and increase the levels of intraplaque hemorrhage incidence[10]. Previous attempts to characterize mast cells and their protease content in human atheromata, by the means of immunohistochemistry, has revealed that they comprise a very heterogeneous population; the majority contain only tryptase, while a smaller but significant proportion contains chymase as well[11]. This heterogeneity is particularly interesting considering that diversity in the quality and quantity of the mast cell degranulating material may exert differential effects inside the atherosclerotic plaque. For example, while mast cell chymase seems to impede tissue repair after a myocardial infarction episode[12], tryptase has been reported to participate in the healing process[13]. Furthermore, mast cells inside human plaques have been found to correlate with typical atherogenic immune populations like dendritic cells and T cells[14]. Importantly, in a human study of 270 patients, intraplaque mast cells emerge as the primary immune cell type to positively associate with future cardiovascular events[15]. Until this day, the means by which mast cells get activated inside the atherosclerotic plaque have not been elucidated in full detail. However, it is suggested that the classical IgE-sensitized pathway[16,17] is involved up to a certain extent. Nevertheless, there is increasing evidence that additional atherosclerosis-specific mechanisms can trigger mast cell activation, independently of IgE-binding[18,19] such as through TLRs[20], complement[21] or neuropeptide[22] receptors. Thus far, the proportional effect of these distinct pathways involved in atherosclerosis, as well as the exact phenotype of mast cells inside human plaques, have not been clarified.

In this technical report of an ongoing cohort study, we made use of the flow

cytometry method to phenotypically characterize human intraplaque mast cells. The atherosclerotic plaque material of 22 human subjects was collected post-operatively from carotid and femoral arteries. Specifically, anonymous individuals, for whom we did not receive any patient details, underwent endarterectomy surgery in a period between July and December 2016 at the Haaglanden Medical Center (HMC), Westeinde, The Hague, NL, after which the atherosclerotic plaque was collected. The handling of all human samples complied with the "Code for Proper Secondary Use of Human Tissue". Human plaques were processed into single cell suspensions by a 2-hour digestion round at 37°C, with an enzyme mix consisting of collagenase IV (Gibco) and DNase (Sigma) as previously described[23]. Subsequently, the samples were filtered through a 70μm cell strainer to obtain single cells. The cells were further stained with extracellular antibodies containing a fluorescent label or fixated and permeabilized (BD Biosciences) for intracellular staining (**Table 1**). Fluorescently labeled samples were measured on a FACS Canto II (BD Biosciences) or Cytoflex (Beckman Coulter) and analyzed using FlowJo software. All data are depicted using GraphPad Prism 7. Values were tested for normalcy. Upon non-Gaussian distribution, an unpaired Mann Whitney *U*-test was performed. In the case of more than two groups a one way-ANOVA analysis was used. Differences lower than $P<0.05$ were considered statistically significant.

Antibody	Fluorochrome	Clone	Concentration	Company
Fixable Viability Dye	eFluor 780	-	0.1μg/sample	eBioscience
CD45	PB	2D1	0.25μg/sample	eBioscience
FcεRIα	APC/PE Cy7	AER-37	0.12 μg/sample	eBioscience
CD117	PercP Cy5.5	104D2	0.1μg/sample	eBioscience
CD63	PE	H5C6	0.1μg/sample	eBioscience
IgE	PE Cy7/FITC	Ige21	0.25μg/sample	eBioscience
Chymase/CMA1	*Alexa Fluor 647*	-	0.1μg/sample	Bioss Antibodies
Tryptase/TPSAB1	*PE*	-	0.1μg/sample	LSBio

Table 1: List of extracellular and *intracellular* antibodies used.

In **Figure 1A** we demonstrate the gating strategy that we followed to detect human intraplaque mast cells. Specifically, we pre-selected all cells from the debris present in the human plaques, based on their size (FSC) and granularity (SSC). Of these, single cells were further separated according to their width (FSC-W) and area (FSC-A). In addition, viability was detected according to the negative binding of a fluorescent viability dye (FVD^{-}). Viable white blood cells were separated based on the expression of the panleukocyte marker CD45. Of these cells, we were able to detect the population of intraplaque mast cells according to the high expression of their characteristic markers FcεRIα, the ligand for IgE[24] and of CD117, the receptor for stem cell factor, a growth factor that is required for the end-stage maturation of mast cells[25]. Accordingly, we observed

that the percentage of mast cells, out of all leukocytes present inside atherosclerotic plaques, is 1.19±0.31% for carotid arteries (n=9) and 1.32±0.21% for femoral arteries (n=13) (**Figure 1B**). Of note, a number of leukocytes may be contained in the excluded debris material. Nonetheless we enumerated the viable cells after manual quantification using both Trypan Blue and in relation to the percentage of viable, CD45$^+$ cells observed according to our gating strategy. We detected that carotid plaques consisted of a mean 12.386±4.961 mast cells while femoral plaques contained 94.273±37.420 mast cells. We were able to isolate more inflammatory cells from femoral as compared to carotid arteries, and thus we identified increased mast cell numbers in femoral plaques (P=0.081; **Figure 1C**). Indeed, femoral plaque samples overall tended to show higher total leukocyte content as compared to carotid plaques (carotid: $12*10^5±4.7*10^5$ leukocytes vs femoral:$76*10^5±27*10^5$ leukocytes, P=0.081; **Figure 1D**).

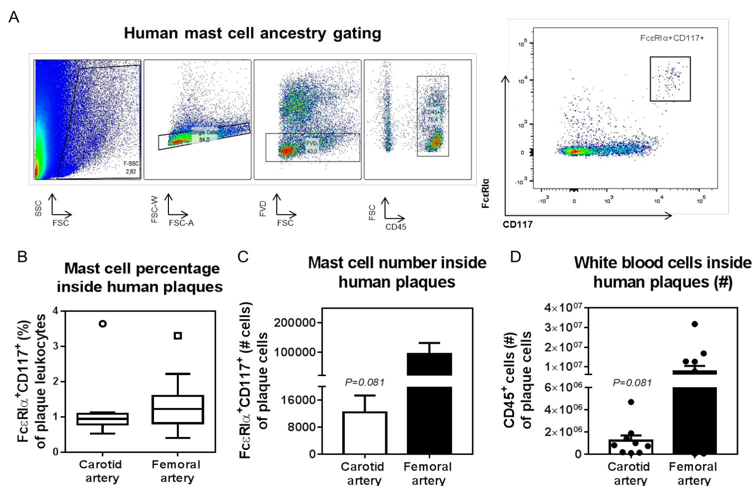

Figure 1: Mast cell content in human plaques. (**A**) Human mast cell gating strategy using flow cytometry. Human intraplaque cells were selected based on their size and area. Viable cells were further separated according to the negative incorporation of fluorescent viability dye (FVD$^-$). Immune cells were detected using the pan-leukocyte marker CD45$^+$ and the human mast cell population was further classified using antibodies against their characteristic markers FcεRIα$^+$ and CD117$^+$. (**B**) Mast cell percentage inside human plaques isolated upon endarterectomy surgeries in carotid and femoral arteries. (**C**) Mast cell absolute numbers inside human carotid and femoral artery samples. The femoral arteries show a slight increase in mast cell content. (**D**) Femoral artery plaques show an increased number of white blood cells as compared to the carotid artery plaques, based on the expression of marker CD45. *Percentages are depicted in a Tukey box plot. Cell numbers are depicted as mean±SEM; (n=10-12/grp).*

We proceeded to characterize the status of the detected human intraplaque cells. Specifically, we stained them for the levels of IgE bound on their surface and observed that inside carotid arteries: 7.779±3.419 intraplaque mast cells, or approximately 63% of the total mast cell population, contained IgE, whereas in the femoral plaques

77.493±35.927 mast cells, or 82% of all mast cells had IgE on their surface (P=0.058; **Figure 2A**). Because IgE binding usually implies mast cell degranulation, we also screened our cells for the expression of CD63, a lysosomal protein that fuses with the membrane upon release of cellular content and therefore marks mast cell activation[26]. We detected 6.998±3.530 $CD63^+$ mast cells in carotid plaques, which indicates about 56% of these cells being in an activated state, while femoral plaques showed 57.417±23.152 activated mast cells, at a total percentage of 61% (**Figure 2B**). We were particularly interested to determine the proportion of IgE mediated activation per patient. For that reason, we analyzed within each arterial plaque sample the population of mast cells that showed IgE bound and also expressed CD63 (IgE^+CD63^+), as opposed to only IgE-binding (IgE^+CD63^-) and only CD63 expression (IgE^-CD63^+) (**Figure 2C**). We discovered that 23.81±3.7% of all human plaque mast cells have IgE bound on their surface without being activated, whereas the majority of mast cells, with 40.01±3.9%, appeared to have IgE bound on their surface and had also undergone degranulation. Interestingly, a proportion of mast cells, namely 19.6±2.9%, appeared to be activated without showing any IgE-fragments bound on their surface, suggesting that this mast cell fraction had been activated *via* alternative mast cell activation pathways. The IgE-activated population was, however, significantly higher than the cells that were subjected to non-IgE mediated activation (P=0.0005), but also higher than the mast cell population that showed binding of IgE without being activated (P=0.0067).

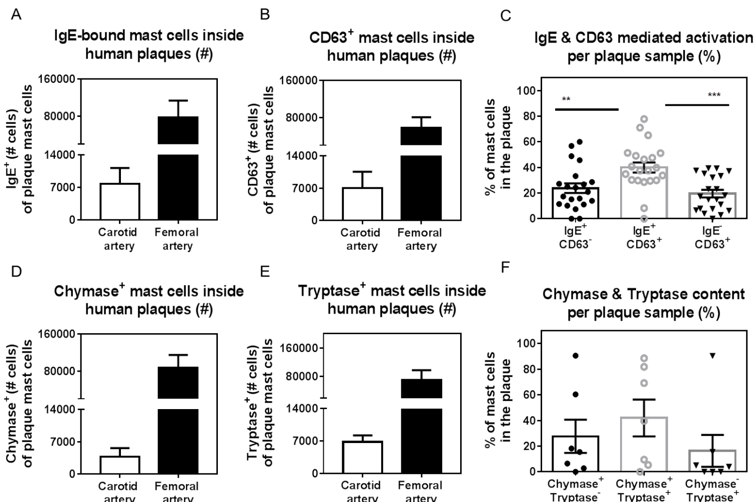

Figure 2: Basic characterization of human intraplaque mast cells. (**A**) IgE-antigen bound mast cells within the atherosclerotic plaques of human carotid and femoral arteries. (**B**) Number of activated mast cells, as defined by marker CD63, inside carotid and femoral artery human plaques. (**C**) Percentage of IgE-bound, IgE-activated and non-IgE activated mast cells per artery sample (n=22). (**D**) Chymase-containing mast cell numbers in human carotid and femoral plaques.(**E**) Number of mast cells containing tryptase in human carotid and femoral arteries. (**F**) Chymase-only, chymase and tryptase-containing and, tryptase-only mast cell percentages per artery sample (n=7). *All values are depicted as mean±SEM. **P<0.01, ***P<0.001*

In a smaller sample of the same patients (n=7) we quantified the mast cell populations that contained chymase and tryptase. Regarding chymase expression, we detected 3.769±1.847 mast cells (30%) inside carotid plaques, and 86.805±28.025 mast cells (92%) inside femoral plaques containing this protease (**Figure 2D**). On the other hand, 6.807±1.439 (54%) of carotid intraplaque mast cells, and 70.627±27.340 (75%) of femoral intraplaque mast cells contained tryptase (**Figure 2E**). Aiming to assess the distribution of chymase and tryptase contents within the mast cell population of the same patients, we analyzed the mast cells consisting of only chymase (Chymase$^+$Tryptase$^-$) or only tryptase (Chymase$^-$Tryptase$^+$), as compared to the mast cells containing both proteases (Chymase$^+$Tryptase$^+$) (**Figure 2F**). We observed that the majority of mast cells, namely 42.0±14.4% were positive for both proteases, while 27.7±13% contained only chymase and 16.4±12.5% only tryptase.

Overall, in this report we are the first to characterize intraplaque mast cells in human atherosclerosis. We provide evidence that the majority of mast cells present in arteries with advanced atherosclerosis are activated through IgE binding, while a smaller fraction can undergo non-IgE dependent activation. We also show that, as mentioned previously[11], human intraplaque mast cells mainly contain both chymase and tryptase, while we detected smaller percentages of either chymase-only, or tryptase-only carrying mast cells; thus confirming the concept of high heterogeneity in the protease content of mast cells in atherosclerotic disease.

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Interestingly, while IgE levels in the circulation[17] have been linked to an increased incidence of acute cardiovascular events and IgE fragments have been previously reported inside human atheromatic tissue[16], up to now it was not clear to which extent this pathway affects the activation of mast cells in the area. Our data confirm that most mast cells present in atherosclerotic plaques are activated[5,10], as it has been shown previously specifically for the shoulder region. Yet, what we show here is that the main activating mechanism is through classical antigen-sensitized IgE binding on their surface Fcε-receptors. Nonetheless, this does not seem to be the only way by which mast cells are activated in human atherosclerosis. It is still unclear what this implies in terms of mediator release. It would be interesting to further characterize the intracellular content of these cells to examine if different activation pathways lead to the release of different proteases and cytokines, and how this may possibly affect the surrounding environment. In addition, we show that a small proportion of cells bind IgE without undergoing activation. Circulating IgE can thus bind on the surface of intraplaque mast cells and sensitize them prior to antigen binding. The exact antigenic fragment that may cause intraplaque mast cell activation has not yet been identified but binding of lipid specific antigenic fragments is the most plausible idea[20]. Furthermore, the detection of IgE fragments inside the plaque tissue confirms that these fragments

can surpass the endothelial wall barrier and accumulate in the plaque area, which may explain the reason why circulating IgE levels correlate with end-stage cardiovascular events like atherothrombosis[27] and myocardial infarction[28]. It is thus reasonable to acknowledge IgE as an important risk factor in cardiovascular episodes, even though it still remains to be elucidated whether it is a causative element[29]. In addition, our data raise an interesting question regarding patients who suffer from other syndromes with increased circulating IgE levels. The development of atherosclerosis is a chronic process, which spans from the formation of a fatty streak, during an individual's teenage years and may result in an unfortunate acute event of an end-stage plaque rupture and vessel occlusion[30]. In the course of those years a fraction of humans may be diagnosed with allergic inflammatory conditions, associating with high levels of circulating IgE[31]. This may mean that IgE has an increased chance to migrate through the endothelium and bind intraplaque mast cells, raising thus the likelihood for a future cardiovascular event. In fact, there is compiling evidence that allergic asthma and atherosclerosis are linked[32], and mast cells are seemingly paramount in this[32]. In addition, patients with a genetic condition called hyper-IgE syndrome have been very recently demonstrated to show signs of coronary subclinical atherosclerosis[33]. Interestingly, our group has demonstrated that the mast cell stabilizer cromolyn acts in a protective manner in atherosclerosis experimental studies[34]. Therefore, a mast cell stabilization approach may be an interesting preventive strategy in individuals who show high circulating IgE levels, but who have not yet been diagnosed with cardiovascular disease syndromes.

As mentioned before, mast cell activation implies release of their granular material. When it comes to their protease secretome, chymase and tryptase are widely acclaimed as the most abundant as well as the most characteristic components of mast cell granules[35]. In our study, human atherosclerotic tissue mast cells show remarkable diversity in their chymase and tryptase content. Experimental mouse models and human observations have previously disclosed the adverse effects exerted by each individual protease in cardiovascular syndromes and suggest specific protease inhibition as a beneficial strategy[36]. One must keep in mind however, that chymase and tryptase can act differentially in the surrounding microenvironment, and that the balance between the two may differently affect plaque characteristics. It seems that while they appear to enhance inflammation at a full-blown or chronic degranulation scale, they can also, under circumstances, promote wound healing[37]. It is important to also note here that the number of patient samples in this analysis was limited as to draw any firm conclusions on the exact protease content and release. Nevertheless, we confirmed that most intraplaque mast cells in humans contain both tryptase and chymase, with an opening for additional studies on human intraplaque mast cells and their secretome. A possible analysis in the concentration of chymase and tryptase levels within the plaque or in the blood of these patients may shed more light into the degranulating material of

these cells.

In conclusion, in this brief human analysis we made use of flow cytometry or the first time, to characterize mast cells finside the atherosclerotic plaques of humans. We confirm that the major pathway for the activation of mast cells inside the plaque tissue is IgE-mediated and that intraplaque mast cells are highly activated and vary in their protease content. This is particularly important since it suggests that already available modes of therapy like mast cell stabilization agents[38], or anti-IgE treatment[39], may prove beneficial in patients suffering from atherosclerosis. In the future, it would be intriguing to assess the plaque stage of these human samples, to gain more information on the exact function of this interesting cell type. We hope that bigger scale patient studies and analysis of more characterization markers, for instance through mass spectrometry, will reveal new pathways *via* which mast cells may act in atherosclerosis, opening thus new ways to interfere in cardiovascular disease.

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