Published in final edited form as:

Andrology. 2014 September ; 2(5): 755–762. doi:10.1111/j.2047-2927.2014.00251.x.

Mononuclear phagocytes rapidly clear apoptotic epithelial cells in the proximal epididymis

T. B. Smith^{1,2}, V. Cortez-Retamozo², L. S. Grigoryeva^{1,2}, E. Hill^{1,2}, M. J. Pittet², and N. Da Silva^{1,2}

¹Division of Nephrology, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

²Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

SUMMARY

We have shown previously that a network of mononuclear phagocytes (MPs) expressing macrophage and dendritic cell markers such as CD11c, F4/80 and CX3CR1, lines the base of the epididymal tubule. However, in the initial segment (IS) and only in that particular segment, epididymal MPs establish extremely close interactions with the epithelium by projecting slender dendrites between most epithelial cells. We undertook the present study to determine how epididymal phagocytes respond to the transient wave of apoptosis initiated by unilateral efferent duct ligation (EDL) in the epididymal epithelium. We show profound morphological and phenotypical changes restricted to the MPs populating the proximal epididymis following EDL. Within 48 h, a large subset of IS epithelial cells had entered an apoptotic state, visualized by the

© 2014 American Society of Andrology and European Academy of Andrology

Correspondence: Nicolas Da Silva, Division of Nephrology and Program in Membrane Biology, Center for Systems Biology, Massachusetts General Hospital, 185 Cambridge Street, CPZN 8.218, Boston, MA 02114-2790, USA. dasilva.nicolas@mgh.harvard.edu.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

N.D.S., T.B.S., V.C.R., L.S.G. and E.H. performed the research. M.J.P. gave conceptual advice and commented on the manuscript. N.D.S. and T.B.S. wrote the paper.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. High-resolution version of Figure 1 panels B and D. Bars = 1 mm.

Figure S2. Unilateral EDL was performed on a CD11c-EYFP mouse, and epididymides were fixed 6 h after the procedure. This low-magnification composite picture of a proximal epididymis section shows that the intensification of YFP that followed EDL fluorescence was restricted to the initial segment (arrow). Bar = 1 mm.

Figure S3. Unilateral EDL was performed on a CD11c-EYFP mouse, and epididymides were fixed 48 h after the procedure. This composite picture of a whole epididymis section shows that the intensification of YFP fluorescence that followed EDL was restricted to the initial segment. Mononuclear phagocytes were not visibly affected by EDL in caput, corpus and cauda epididymis. Bar = 1 mm.

Figure S4. Cell cycle analysis of epididymis samples by flow cytometry confirmed that, 48 h after EDL, apoptotic cells are present primarily in the proximal epididymis (region 1). The 'sub-G1' peak (horizontal bars) is increased by a factor of 6.5 in the region that contains the initial segment, while it's only doubled in region 2, and stable in the distal epididymis (region 3). Error bars represent the standard error of the mean.

Movie clips S1 and S2. 3D rendering of the z stacks used for Fig. 4a, panels F,G, representing epithelial nuclei engulfed by CD11c⁺ MPs 6 h after EDL.

Movie clip S3. 3D rendering of a z stack representing a cross-section of the epididymal tubule in the initial segment of a CD11c-EYFP (green) mouse, fixed 48 h after EDL, and immunolabelled with an anti-ZO-1 antibody to reveal tight junctions (red). Nuclei are labelled with DAPI (blue).

terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay and CD11c⁺ and CX3CR1⁺ MPs readily engulfed TUNEL-positive cells and other debris. Despite the high levels of apoptosis and the rapid clearance of apoptotic cells occurring after EDL, the epithelium preserved its overall architecture and maintained tight junctions of the blood–epididymis barrier (BEB). The discovery of a functional population of MPs in the epididymal epithelium responsible for maintaining the integrity of the BEB raises further questions regarding the role of these cells in clearing defective epithelial cells in the steady-state epididymis, as well as pathogens and abnormal spermatozoa in the lumen.

Keywords

animal models; apoptosis; epididymis; immunology; mouse; reproductive tract

INTRODUCTION

The epididymis is the primary site of sperm maturation and storage (Robaire *et al.*, 2006; Cornwall, 2009; Shum *et al.*, 2011; Belleannee *et al.*, 2012; Dacheux & Dacheux, 2013). Unlike other organs such as the testis and kidney, which contain numerous seminiferous tubules and nephrons, respectively, the epididymis is reliant on the uninterrupted physical and functional integrity of a single, long and convoluted tubule in order to establish and maintain male fertility. Any obstruction of the lumen or alteration of the epididymal epithelium has the potential to affect fertility transiently or permanently, for instance, by breaching the barrier that is assumed to physically isolate antigenic spermatozoa from the immune system and thus to prevent the production of antisperm antibodies. Accordingly, the value of highly efficient mechanisms for epithelial maintenance in the epididymis is self-evident.

The epididymal duct is lined by a pseudostratified epithelium, essentially composed of principal, clear and basal cells, and is surrounded by cells from the mononuclear phagocyte system (macrophages and dendritic cells) (Robaire & Hermo, 1988; Turner, 1995; Flickinger *et al.*, 1997; Serre & Robaire, 1999; Hermo & Robaire, 2002; Robaire & Hinton, 2002; Robaire *et al.*, 2006; Da Silva *et al.*, 2011; Arrighi, 2013; Shum *et al.*, 2014). The different epithelial cell subtypes, connected by tight junctions and surrounded by immune cells, constitute altogether the highly efficient ‘blood–epididymis barrier’ (BEB) that allows spermatozoa to mature and be stored in an optimal immunological microenvironment (Hoffer & Hinton, 1984; Wu *et al.*, 1986; Dube *et al.*, 2010; Mital *et al.*, 2011). Unsurprisingly, testicular factors, including androgens, play a critical role in the development of the epididymis as well as in the regulation of its functions (Nicander *et al.*, 1983; Fan & Robaire, 1998; Turner & Riley, 1999). Efferent duct ligation (EDL) is a minimally invasive procedure which has revealed an abundance of information regarding the importance of luminal testicular factors in epididymal physiology, achieved by blocking the flow of luminal fluid into the epididymis without significant disruption of blood flow and, therefore, systemic endocrine regulation. In the most proximal portion of the epididymis, designated ‘initial segment’ in the rat and in the mouse, principal cells are predominantly dependent on luminal factors for survival, owing to the observation that a significant subset

of principal cells enter apoptosis shortly after the disruption of luminal flow as a result of EDL (Nicander *et al.*, 1983; Turner & Riley, 1999). Despite the fact that this rapid and extensive loss of epithelial cells is expected to lead to a partial destruction of the epithelial surface, the histological data published to date indicate that the overall morphology of the tubule is relatively well preserved (Abe & Takano, 1989a,b; Robaire & Fan, 1998; Turner & Riley, 1999; Turner *et al.*, 2003, 2007a). The IS-specific wave of apoptosis is transient, indicating that a subset of cells has the ability to survive in the post- EDL environment and readily replace apoptotic androependent epithelial cells. However, the regenerated proximal epididymis regresses to a transcriptionally undifferentiated state (Turner *et al.*, 2007a,b). To date, the mechanisms that allow the proximal epididymis to survive this massive wave of cell death while preserving the integrity of the tubule have not been investigated.

In homeostatic conditions, apoptosis plays a critical role in the development and maintenance of most tissues. Cell corpses and debris are harmful for the surrounding healthy cells, and therefore the maintenance of epithelia requires not only the proper execution of the apoptotic program but also efficient removal of dead cells and debris. In addition, clearance of unwanted and apoptotic cells is necessary to prevent the development of malignancies, as well as autoimmune and infectious diseases (Elliott & Ravichandran, 2010; Elliott *et al.*, 2010; Tanaka *et al.*, 2010; Hochreiter-Hufford & Ravichandran, 2013; Ravishankar & McGaha, 2013). The professional effectors of apoptotic clearance are macrophages and dendritic cells and the IS of the epididymis, which is rapidly and profoundly affected by EDL, is also the segment that contains the most intriguing population of mononuclear phagocytes (MPs). We have shown previously that a dense and heterogeneous network of MPs that express several macrophage and dendritic cell markers such as CD11c (integrin alpha X chain), F4/80 and CX3CR1 (fractalkine receptor), lines the base of the entire epididymal tubule (Da Silva *et al.*, 2011). Exclusively in the IS, epididymal MPs establish extremely close interactions with the epithelium, specifically by projecting slender dendritic processes between epithelial cells. We undertook the present study to determine how epididymal phagocytes (eMPs) expressing CD11c and CX3CR1 respond to the disruption of luminal flow induced by EDL. We show that, following EDL, epithelium-associated MPs undergo rapid and massive morphological and phenotypical changes in order to phagocytize apoptotic epithelial cells, revealing a role of CD11c⁺ and CX3CR1⁺ phagocytes as housekeepers of the BEB.

MATERIALS AND METHODS

Mice

CD11c-EYFP mice (Lindquist *et al.*, 2004) and CX3CR1-GFP mice (Jung *et al.*, 2000) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). CD11c is the integrin alpha X chain, encoded by *Itgax*. CX3CR1 is the G protein-coupled fractalkine receptor. Mice referred to as 'CX3CR1-GFP' were *Cx3cr1^{gfp/+}* mice, obtained by breeding *Cx3cr1^{gfp/gfp}* males with C57BL/6 females. The genotype of all mice was confirmed by PCR analysis of tail snip DNA. Transgenic and wild-type mice were maintained free of

common rodent pathogens and on a standard lab chow diet. Mouse protocols were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Efferent duct ligation

Adult mice (at least 70 days old) were anaesthetized with isoflurane and epididymides and testes exposed through a low midline abdominal incision. A 6-0 silk suture was secured firmly around the efferent ducts, avoiding any damage to the neighbouring blood vessels. Epididymides and testes were placed back into the abdomen and the incision was closed. The contralateral side was used as a sham control. Animals were allocated into seven groups (1–7 days post-procedure). At the assigned time, mice were euthanized using an overdose of isoflurane by inhalation, and bilateral epididymides and testes were harvested for analysis.

Post-EDL flow cytometry analysis of eMPs

Twenty C57BL/6J mice per experiment were subjected to unilateral EDL. Forty-eight hours after the procedure, epididymides from the EDL side and the control side were dissected and separated into three regions representing the proximal epididymis (IS and proximal caput, region 1), central epididymis (distal caput and corpus, region 2) and distal epididymis (cauda, region 3) respectively. Individual cell suspensions were prepared as described previously (Da Silva *et al.*, 2011). Briefly, epididymis regions were minced with scissors in a dissociation medium containing collagenase type I and type II and incubated for 45 min at 37 °C with gentle shaking. Cell suspensions were passed through a 70-µm nylon mesh to remove clumps, washed and stored on ice until processing. For negative selection of MPs, cell suspensions were incubated with a cocktail of PE-labelled monoclonal antibodies against T cells, B cells, NK cells and granulocytes, and depleted using anti-PE microbeads and a MACS column (Miltenyi Biotec, Auburn, CA, USA) as previously described (Da Silva *et al.*, 2011). MP-enriched cell suspensions were then labelled with CD11c-Alexa 700 (HL3), CD11b-APC-Cy7 (M1/70), F4/80-PE-Cy7 (BM8), CD103-APC (M290). The experiment was performed twice. All antibodies were purchased from BD Biosciences (San Jose, CA, USA) or eBioscience (San Diego, CA, USA). The percentage of cells with 'sub-G1' DNA was used to evaluate DNA fragmentation and, therefore, apoptosis. In brief, cells were fixed using the Cytotfix/Cytoperm reagent (BD Biosciences) as per the manufacturer's instruction and the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) prior to flow cytometry-based analysis. All flow cytometry data were acquired in triplicate on an LSRII flow cytometer (BD Biosciences). 500,000 events per sample were acquired. Results were analysed with FlowJo 9 (Tree Star, Ashland, OR, USA). Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). $N = 40$ mice. Analysis was performed by paired t -test. Data are presented as mean $\pm$ standard error of the mean (SEM). The level of statistical significance was set at $p < 0.05$.

Epifluorescence and confocal microscopy analyses

Epididymides were fixed by immersion in periodate-lysineparaformaldehyde or 4% paraformaldehyde in phosphate-buffered saline (PBS). Fixed tissues were cryoprotected with 30% sucrose in PBS embedded in optimal cutting temperature compound (Leica Microsystems, Buffalo Grove, IL, USA) and sectioned in a Leica cryostat at a thickness of

10–50 μm . Immunolabelling was performed as described previously (Da Silva *et al.*, 2006) with an anti-ZO-1 antibody (clone R40.76), followed by a Cy3 goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA, USA). TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay for detection of apoptotic cells in situ was performed using the Click-iT TUNEL Imaging kit (Life Technologies, Eugene, OR, USA) following the manufacturer's instructions. Microscopic images were acquired with an Eclipse 90i epifluorescence microscope (Nikon Instruments, Melville, NY, USA). Confocal images were acquired using a Nikon A1R scanning confocal microscope. Digital image files were post-processed with Nikon Imaging Software Elements, Volocity 6 (Perkin Elmer, Waltham, MA, USA), ImageJ, Adobe Photoshop CS6 and Apple QuickTime. The extended depth of field in 2D micrographs (Figs 2, 4A, 5 & 6) was obtained by performing maximum intensity projections of epifluorescence and confocal z stacks from thick tissue sections (25–50 μm).

RESULTS

The overall appearance of the epididymal mononuclear phagocyte network in the proximal epididymis is affected by EDL

Unilateral EDL was performed with CD11c-EYFP and CX3CR1-GFP mice, which allowed for the visualization of mononuclear macrophages in situ. The success of the EDL procedure was confirmed by the rapid clearance of spermatozoa from the lumen of the proximal epididymis (IS and proximal caput). We observed an apparent densification of the MP network in the proximal epididymis, and more specifically in the IS (Fig. 1 and Fig. S1 for high-resolution images). In the IS (highlighted with dotted lines in Fig. 1), the intensity of fluorescence was increased and MPs appeared larger after EDL (Fig. 1B,D) compared to controls (Fig. 1A,C). Although this response peaked 48–72 h after the procedure, an overall increase in fluorescence could be detected as early as 6 h after EDL (Fig. S2). EYFP and GFP are expressed as reporter cytosolic proteins under the control of CD11c and CX3CR1 promoters, respectively, therefore the overall increase in fluorescence could reflect either an increase in the number of CD11c⁺ and CX3CR1⁺ MPs, an increase in the cellular volume, and/or a higher rate of reporter protein expression. Notwithstanding these findings, the more distal regions (distal caput, corpus and cauda) remained unaffected by EDL (Fig. S3), therefore, further microscopic studies focused on the proximal epididymis.

EDL induces profound morphological changes in MPs in the IS

Higher magnification images revealed that, specifically in the IS, EDL dramatically affected MP's morphology. In control epididymides (Fig. 2A,B), MPs were mostly peritubular as described previously (Da Silva *et al.*, 2011). Cellular bodies located at the base of the epithelium and slender dendritic processes extended towards the lumen, between neighbouring epithelial cells. Forty-eight hours after EDL, most CD11c⁺ (Fig. 2B,C) and CX3CR1⁺ (Fig. 2E,F) phagocytes had lost their intraepithelial dendrites, had become globular, were filled with large phagosomes and had migrated more apically into the epithelial layer. Interestingly, counterstaining with Evans blue (Fig. 2B,E) and DAPI (Fig. 2C,F) revealed that the overall structure of the tubule was well maintained. We occasionally detected CD11c⁺ cells within the lumen of the proximal epididymis (visible in Fig. 2C, arrowheads).

The phenotype of MPs is affected by EDL primarily in the IS

Although microscopy studies revealed unequivocal morphological changes of the phagocyte network in the proximal epididymis, we undertook a flow cytometry-based analysis of epididymal cell suspensions to analyze the phenotypical modifications induced by EDL in a more quantitative manner. Twenty mice were subjected to unilateral EDL and, 48 h after the procedure, epididymides from the EDL side and the control side were dissected and separated into three discrete regions representing the proximal epididymis (IS and proximal caput: region 1), central epididymis (distal caput and corpus: region 2) and distal epididymis (cauda: region 3) respectively. As expected, the most noticeable phenotypical changes were observed in region 1 (Fig. 3A). The relative number of CD11b⁺ (blue bar), F4/80⁺ (green bar) and CD11c⁺ cells (orange bar) increased two-fold, indicating that EDL quantitatively altered MP populations in the proximal epididymis. The most affected subset of MPs was CD11b⁺ cells expressing intermediate (int) levels of CD11c and F4/80 (Fig. 3A, red bar). The increased population of CD11b^{high} CD11c^{int} F4/80^{int} cells is shown more precisely in Fig. 3B. The panels to the left show the marked increase in the number of CD11b⁺ cells in the EDL sample compared to control (37% vs. 18%), and panels to the right highlight the increase in F4/80^{int} cells (45% vs. 15%). This increase occurred at the expense of a distinct subset of CD11b⁺ cells that expresses high levels of CD11c and F4/80 (Fig. 3A, purple bar). In contrast, the CD11b⁻ CD103⁺ and CD11b⁺ CD103⁻ subsets increased identically in region 1 following EDL (Fig. 3A, grey and black bars respectively). MPs isolated from region 2 were not significantly affected by EDL, while region 3 showed a two-fold increase in the number of CD11b⁺ cells, and specifically CD11b⁺ cells expressing intermediate levels of F4/80 and CD11c (Fig. 3A, blue and red bars respectively).

MPs rapidly engulf apoptotic epithelial cells in the IS after EDL

The TUNEL assay was performed on epididymal sections in order to visualize the apoptotic nuclei in situ. Forty-eight hours after EDL, TUNEL-positive cells were rare in the efferent ducts, very abundant in the IS and progressively disappeared in the caput region (Fig. 4A). CD11c⁺ cells showing a globular appearance were present exclusively in the IS and co-localized with the TUNEL-positive region (Fig. 4B,C). High-magnification images revealed the presence of TUNEL-positive nuclei inside CD11c⁺ cell phagosomes (Fig. 4D,E), suggesting that MPs actively engulf debris from apoptotic cells in the IS. In sections counterstained with DAPI, dense apoptotic nuclei were also visible in CD11c⁺ phagocytic cells (Fig. 4F,G, and movie clips S1 and S2) 6 h after EDL. In order to correlate the presence of apoptotic cells with the phenotypical changes described in Fig. 3, we quantified the presence of apoptotic nuclei in regions 1–3, revealed by an increase in the percentage of ‘sub-G1’ cells detected by flow cytometry. Analysis of regions 1–3 48 h post-EDL revealed a 6-fold increase in apoptotic cells in region 1 (which contains the IS) and a moderate 2-fold increase in apoptotic nuclei in region 2, whereas region 3 remained unaffected (Fig. S4).

Epithelial tight junctions are preserved after EDL

Epithelial tight junctions (TJs) represent a critical component of the BEB. We analysed the distribution of tight junction protein ZO-1 (zona occludens-1) by immunofluorescence to evaluate the potential damage caused by EDL on the BEB (Fig. 5 and movie clip S3). The

apical TJ network (Fig. 5A, red labelling) was well preserved in the IS despite the presence of numerous apoptotic epithelial cells and extremely abundant intraepithelial phagocytes (green). The higher magnification image of the IS epithelium seen from the apical side (Fig. 5B) confirms that, despite the presence of occasional breaches (arrows), the network of apical tight junctions remained mostly continuous.

Alteration of IS MPs is reversible

Four days after EDL, MPs in the IS returned to a dendriform morphology again (Fig. 6). Bodies from CD11c⁺ (Fig. 6A,C) and CX3CR1 cells (Fig. 6B) relocated in the basal region of the epithelium and dendrites, which were mostly absent during the peak of epithelial apoptosis, again extended between epithelial cells. Notwithstanding the irreversibility of EDL and despite the presence of residual MPs displaying a phagocytic appearance, the MP network returned to a pre-EDL appearance.

DISCUSSION

Here, we show that the intricate network of peritubular CD11c⁺ and CX3CR1⁺ MPs, which we identified previously in the mouse epididymis (Da Silva *et al.*, 2011), are locally involved in the phagocytic clearance of apoptotic epithelial cells. When the rate of epithelial apoptosis was experimentally increased *in vivo* by performing EDL, MPs from the IS underwent striking morphological and phenotypical changes within a short period of time, in order to engulf and eliminate dying cells and debris. As a result, and despite the massive wave of epithelial apoptosis, the overall morphology of the tubule and the integrity of the BEB remained well preserved. Using CD11c-EYFP and CX3CR1-GFP transgenic mice, we show a massive reorganization of the peritubular MP network that correlates well with the epithelial degeneration and subsequent clean up of degenerated cells beautifully described by Abe and Takano 25 years ago (Abe & Takano, 1989a,b). In direct contrast with our results, Seiler *et al.* (1999) observed a decrease in F4/80⁺ cells following EDL, with no significant morphological change. The F4/80⁺ ‘basal cells’ described in the mouse epididymis by this group are without doubt a subset of epididymal MPs. We have shown that F4/80 is expressed by CD11c⁺ CX3CR1⁺ cells, but not by basal cells characterized by keratin 5 expression (Shum *et al.*, 2014). Based on the fact that a significant proportion of principal cells die after EDL, estimated to be as high as 50% in the rat proximal epididymis (Turner & Riley, 1999) because of their inherent dependence on luminal androgens (Fan & Robaire, 1998), and because principal cells represent the most abundant epithelial cell type in the IS (Robaire *et al.*, 2006), we anticipated that a consequence of the damage caused by EDL would be a dramatic decline in the integrity of the BEB. Microscopy and flow cytometry analyses confirmed that massive apoptosis was a feature of the post-EDL epididymal epithelium. However, tight junction protein ZO-1 immunolabelling demonstrated that the physical integrity of the apical tight junction network, which is an essential element of the BEB, is only mildly disrupted by EDL. Our results clearly demonstrate that the abundant peritubular MPs that populate the epididymis engulf and clear apoptotic corpses very efficiently even under the extreme and non-physiological conditions introduced by EDL. This indicates that the proximal epididymis contains a highly efficient

maintenance mechanism despite the relatively slow turnover of epithelial cells (Yeung *et al.*, 2012).

Although EDL is not reversible, IS MPs returned to their steady-state appearance 4–5 days after the procedure. This suggests that the presence of spermatozoa and testicular factors in the luminal compartment is not required to retain the MP network in the epididymis. The presence of IS-specific intraepithelial dendrites seems to be an intrinsic property of this mucosal system in adult mice, regardless of the composition of the lumen and the differentiation status of the epithelium. While luminal hormones produced in the testis do not appear to play a role in the presence and maintenance of MPs in the epididymis, a possible role of circulating hormones remains unexplored. A significant question arising from this study is the requirement to elucidate the role of the abundant population of MPs in the steady-state epididymis. It has been reported that few apoptotic cells are detected in tissues including the thymus, testis and bone marrow regardless of the fact that these tissues experience a high turnover rate. It is only when the mechanisms regulating phagocytic clearance are disrupted that an accumulation of apoptotic corpses is observed (Ravishankar & McGaha, 2013). In keeping with this observation, we hypothesize that the low rate of apoptosis in the epididymal epithelium that has been measured by other groups is partly a consequence of rapid and efficient mechanisms for apoptotic clearance, mediated by peritubular resident MPs constantly monitoring neighbouring epithelial cells. In the steady-state IS, epithelial apoptosis is highly unlikely to cause a breach in the BEB, suggesting again the primary importance of this segment.

Given that each epididymis is composed of a single tubule, prompt and silent epithelial maintenance is of great importance. Phagocytic clearance involves a finely regulated sequence of molecular events aimed at identifying, taking up and eliminating apoptotic cells (Elliott & Ravichandran, 2010; Ravishankar & McGaha, 2013). The failure to clear apoptotic cells before they become secondarily necrotic results in the release of pro-inflammatory cytokines, recruiting inflammatory cells such as neutrophils to the area, resulting in damage to neighbouring cells and initiating immune responses to autoantigens (Hochreiter-Hufford & Ravichandran, 2013). Furthermore, a putative posttesticular ‘sperm quality control’ in the epididymis has been suggested which would require the identification and elimination of defective spermatozoa. Several mechanisms have been suggested for the removal of abnormal spermatozoa, including proteolytic degradation and ‘spermiophagy’ mediated by epithelial cells or leucocytes (Jrad-Lamine *et al.*, 2011). A number of molecular and cellular factors involved in apoptotic cell clearance, as well as in immune tolerance have been identified in the epididymis, including MFG-E8 [milk fat globule epidermal growth factor (EGF) factor 8] (Raymond & Shur, 2009; Raymond *et al.*, 2009, 2010), IDO (Jrad-Lamine *et al.*, 2011, 2013), and several subsets of antigen-presenting cells and phagocytes (Da Silva *et al.*, 2011). The exact function of these cells and factors often remains elusive, however. The IS constitutes the ultimate section of the male reproductive tract in which the diluted spermatozoa have the opportunity to interact directly with an epithelium heavily infiltrated by immune cells with potent phagocytic and antigen-presenting functions. The factors that regulate interactions between immune cells, epithelial cells and spermatozoa remain to be clearly identified, however, one previously identified protein has the potential

to play a central role. The adhesive molecule MFG-E8 is secreted in the IS of the mouse epididymis where it coats the surface of spermatozoa to become a regulator of spermatozoa–egg interactions (Ensslin & Shur, 2003). Furthermore, MFG-E8 is a well-described bridging molecule that facilitates the engulfment of apoptotic cells by phagocytes by binding to phosphatidylserine, and has also been involved in the maintenance of the epididymal epithelium (Raymond & Shur, 2009; Raymond *et al.*, 2009, 2010). MFG-E8 has, therefore, the potential to be a major regulator of interactions between eMPs, epithelial cells and possibly spermatozoa. Interestingly, apoptotic spermatozoa from humans have been found to die via a truncated apoptotic pathway mediated by caspases and culminating in the externalization of phosphatidylserine, the universal marker of apoptosis and a prerequisite for recognition by phagocytes (Koppers *et al.*, 2011; Hochreiter-Hufford & Ravichandran, 2013). The role of peritubular phagocytes in the uptake of apoptotic spermatozoa in the lumen requires further investigation as the externalization of phosphatidylserine might also be a signal for the silent phagocytosis of apoptotic spermatozoa in the female reproductive tract.

Our present work shows that the epididymis constitutes a remarkable model to study phagocytic clearance *in vivo*: a simple surgical procedure triggers epithelial apoptosis in a rapid, local and transient manner with respect to the overall structure of the organ. Furthermore, the rapid replacement of apoptotic, lumicrine factor-dependent principal cells with an uncharacterized subset of principal cells capable of surviving under lumicrine-depleted conditions remains intriguing. Overall, EDL constitutes an interesting *in vivo* model of epithelial regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This study was supported by National Institutes of Health grants R01HD069623 (NDS) and R01AI084880 (MJP). The Microscopy Core facility of the MGH Program in Membrane Biology receives support from the Boston Area Diabetes and Endocrinology Research Center (DK057521) and the MGH Center for the Study of Inflammatory Bowel Disease (DK043351).

REFERENCES

- Abe K, Takano H. Early degeneration of the epithelial cells in the initial segment of the epididymal duct in mice after efferent duct cutting. *Arch Histol Cytol.* 1989a; 52:299–310. [PubMed: 2505829]
- Abe K, Takano H. Cytological response of the principal cells in the initial segment of the epididymal duct to efferent duct cutting in mice. *Arch Histol Cytol.* 1989b; 52:321–326. [PubMed: 2513843]
- Arrighi S. Are the basal cells of the mammalian epididymis still an enigma? *Reprod Fertil Dev.* 2013
- Belleannee C, Thimon V, Sullivan R. Region-specific gene expression in the epididymis. *Cell Tissue Res.* 2012; 349:717–731. [PubMed: 22427067]
- Cornwall GA. New insights into epididymal biology and function. *Human Reprod Update.* 2009; 15:213–227.
- Da Silva N, Silberstein C, Beaulieu V, Pietrement C, Van Hoek AN, Brown D, Breton S. Postnatal expression of aquaporins in epithelial cells of the rat epididymis. *Biol Reprod.* 2006; 74:427–438. [PubMed: 16221990]

- Da Silva N, Cortez-Retamozo V, Reinecker HC, Wildgruber M, Hill E, Brown D, Swirski FK, Pittet MJ, Breton S. A dense network of dendritic cells populates the murine epididymis. *Reproduction*. 2011; 141:653–663. [PubMed: 21310816]
- Dacheux JL, Dacheux F. New insights into epididymal function in relation to sperm maturation. *Reproduction*. 2013; 147:R27–R42. [PubMed: 24218627]
- Dube E, Hermo L, Chan PT, Cyr DG. Alterations in the human blood-epididymis barrier in obstructive azoospermia and the development of novel epididymal cell lines from infertile men. *Biol Reprod*. 2010; 83:584–596. [PubMed: 20505168]
- Elliott MR, Ravichandran KS. Clearance of apoptotic cells: implications in health and disease. *J Cell Biol*. 2010; 189:1059–1070. [PubMed: 20584912]
- Elliott MR, Zheng S, Park D, Woodson RI, Reardon MA, Juncadella JJ, Kinchen JM, Zhang J, Lysiak JJ, Ravichandran KS. Unexpected requirement for ELMO1 in clearance of apoptotic germ cells in vivo. *Nature*. 2010; 467:333–337. [PubMed: 20844538]
- Ensslin MA, Shur BD. Identification of mouse sperm SED1, a bimotif EGF repeat and discoidin-domain protein involved in sperm-egg binding. *Cell*. 2003; 114:405–417. [PubMed: 12941270]
- Fan X, Robaire B. Orchidectomy induces a wave of apoptotic cell death in the epididymis. *Endocrinology*. 1998; 139:2128–2136. [PubMed: 9529002]
- Flickinger CJ, Howards SS, Baran ML, Pessoa N, Herr JC. Appearance of ‘natural’ antisperm autoantibodies after sexual maturation of normal Lewis rats. *J Reprod Immunol*. 1997; 33:127–145. [PubMed: 9234212]
- Hermon L.; Robaire B. Epididymal cell types and their functions. In: Robaire, B.; Hinton, BT., editors. *The Epididymis: From Molecular to Clinical Practice A Comprehensive Survey of the Efferent Ducts, the Epididymis and the Vas Deferens*. New York: Kluwer Academic/Plenum; 2002. p. 81-102.
- Hochreiter-Hufford A, Ravichandran KS. Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion. *Cold Spring Harb Perspect Biol*. 2013; 5:a008748. [PubMed: 23284042]
- Hoffer AP, Hinton BT. Morphological evidence for a blood-epididymis barrier and the effects of gonadotropin on its integrity. *Biol Reprod*. 1984; 30:991–1004. [PubMed: 6733204]
- Jrad-Lamine A, Henry-Berger J, Goubeyre P, Damon-Soubeyrand C, Lenoir A, Combaret L, Saez F, Kocer A, Tone S, Fuchs D, Zhu W, Oefner PJ, Munn DH, Mellor AL, Gharbi N, Cadet R, Aitken RJ, Drevet JR. Deficient tryptophan catabolism along the kynurenine pathway reveals that the epididymis is in a unique tolerogenic state. *J Biol Chem*. 2011; 286:8030–8042. [PubMed: 21189261]
- Jrad-Lamine A, Henry-Berger J, Damon-Soubeyrand C, Saez F, Kocer A, Janny L, Pons-Rejraji H, Munn DH, Mellor AL, Gharbi N, Cadet R, Guiton R, Aitken RJ, Drevet JR. Indoleamine 2,3-dioxygenase 1 (ido1) is involved in the control of mouse caput epididymis immune environment. *PLoS ONE*. 2013; 8:e66494. [PubMed: 23840489]
- Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, Littman DR. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol*. 2000; 20:4106–4114. [PubMed: 10805752]
- Koppers AJ, Mitchell LA, Wang P, Lin M, Aitken RJ. Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxidative DNA damage in human spermatozoa. *Biochem J*. 2011; 436:687–698. [PubMed: 21470189]
- Lindquist RL, Shakhbar G, Dudziak D, Wardemann H, Eisenreich T, Dustin ML, Nussenzweig MC. Visualizing dendritic cell networks in vivo. *Nat Immunol*. 2004; 5:1243–1250. [PubMed: 15543150]
- Mital P, Hinton BT, Dufour JM. The blood-testis and blood-epididymis barriers are more than just their tight junctions. *Biol Reprod*. 2011; 84:851–858. [PubMed: 21209417]
- Nicander L, Osman DI, Ploen L, Bugge HP, Kvisgaard KN. Early effects of efferent ductule ligation on the proximal segment of the rat epididymis. *Int J Androl*. 1983; 6:91–102. [PubMed: 6840881]
- Ravishankar B, McGaha TL. O death where is thy sting? Immunologic tolerance to apoptotic self. *Cell Mol Life Sci*. 2013; 70:3571–3589. [PubMed: 23377225]

- Raymond AS, Shur BD. A novel role for SED1 (MFG-E8) in maintaining the integrity of the epididymal epithelium. *J Cell Sci.* 2009; 122:849–858. [PubMed: 19240116]
- Raymond A, Ensslin MA, Shur BD. SED1/MFG-E8: a bi-motif protein that orchestrates diverse cellular interactions. *J Cell Biochem.* 2009; 106:957–966. [PubMed: 19204935]
- Raymond AS, Elder B, Ensslin M, Shur BD. Loss of SED1/ MFG-E8 results in altered luminal physiology in the epididymis. *Mol Reprod Dev.* 2010; 77:550–563. [PubMed: 20422713]
- Robaire B, Fan X. Regulation of apoptotic cell death in the rat epididymis. *J Reprod Fertil Suppl.* 1998; 53:211–214. [PubMed: 10645279]
- Robaire, B.; Hermo, L. Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. In: Knobil, E.; Neil, J., editors. *The Physiology of Reproduction*. New York: Raven Press; 1988. p. 999-1080.
- Robaire, B.; Hinton, BT. The epididymis: From molecular to clinical practice. A comprehensive survey of the efferent ducts, the epididymis and the vas deferens. New York: Kluwer Academic/ Plenum; 2002.
- Robaire, B.; Hinton, BT.; Orgebin-Crist, MC. The epididymis. In: Neill, JD., editor. *Knobil and Neill's Physiology of Reproduction*. New York: Elsevier Academic Press; 2006. p. 1072-1148.
- Seiler P, Cooper TG, Yeung CH, Nieschlag E. Regional variation in macrophage antigen expression by murine epididymal basal cells and their regulation by testicular factors. *J Androl.* 1999; 20:738–746. [PubMed: 10591613]
- Serre V, Robaire B. Distribution of immune cells in the epididymis of the aging Brown Norway rat is segment-specific and related to the luminal content. *Biol Reprod.* 1999; 61:705–714. [PubMed: 10456848]
- Shum WW, Ruan YC, Da Silva N, Breton S. Establishment of cell-cell cross talk in the epididymis: control of luminal acidification. *J Androl.* 2011; 32:576–586. [PubMed: 21441423]
- Shum WW, Smith TB, Cortez-Retamozo V, Grigoryeva LS, Roy JW, Hill E, Pittet MJ, Breton S, Da Silva N. Epithelial basal cells are distinct from dendritic cells and macrophages in the mouse epididymis. *Biol Reprod.* 2014; 90:1–10.
- Tanaka M, Asano K, Qiu CH. Immune regulation by apoptotic cell clearance. *Ann N Y Acad Sci.* 2010; 1209:37–42. [PubMed: 20958314]
- Turner TT. On the epididymis and its role in the development of the fertile ejaculate. *J Androl.* 1995; 16:292–298. [PubMed: 8537245]
- Turner TT, Riley TA. p53 independent, region-specific epithelial apoptosis is induced in the rat epididymis by deprivation of luminal factors. *Mol Reprod Dev.* 1999; 53:188–197. [PubMed: 10331457]
- Turner TT, Bomgardner D, Jacobs JP, Nguyen QA. Association of segmentation of the epididymal interstitium with segmented tubule function in rats and mice. *Reproduction.* 2003; 125:871–878. [PubMed: 12773110]
- Turner TT, Johnston DS, Finger JN, Jelinsky SA. Differential gene expression among the proximal segments of the rat epididymis is lost after efferent duct ligation. *Biol Reprod.* 2007a; 77:165–171. [PubMed: 17377138]
- Turner TT, Johnston DS, Jelinsky SA, Tomsig JL, Finger JN. Segment boundaries of the adult rat epididymis limit interstitial signaling by potential paracrine factors and segments lose differential gene expression after efferent duct ligation. *Asian J Androl.* 2007b; 9:565–573. [PubMed: 17589796]
- Wu YW, Yuan D, Xie YK. Electrophysiological and ultrastructural studies of the blood-epididymis barrier of the rat. *Shi Yan Sheng Wu Xue Bao.* 1986; 19:293–303. [PubMed: 3577546]
- Yeung CH, Wang K, Cooper TG. Why are epididymal tumours so rare? *Asian J Androl.* 2012; 14:465–475. [PubMed: 22522502]

Figure 1.

The mononuclear phagocyte network is affected by EDL in the initial segment of the mouse epididymis. Unilateral EDL was performed on CD11c-EYFP and CX3CR1-GFP mice. Forty-eight hours after the procedure, epididymis sections were analysed by fluorescence microscopy. These low-magnification pictures show an intensification of the CD11c- and CX3CR1-driven fluorescence after EDL (B, D) in the most proximal region of the epididymis, compared to the most distal segments and to unoperated controls (A, C), indicating that the size and/or the number of peritubular CD11c⁺ and CX3CR1⁺ cell was increased subsequent to the blockade of luminal flow upstream of the epididymis. Bars = 1 mm. High-resolution pictures are available online (Fig. S1).

Figure 2.

Efferent duct ligation (EDL) induces marked morphological changes in mononuclear phagocytes (MPs) in the initial segment (IS). Unilateral EDL was performed in CD11c-EYFP and CX3CR1-GFP mice. Epididymides were dissected and fixed 48 h after the procedure, and sections were analysed by fluorescence microscopy. Panels A and B show the morphology of CD11c⁺ and CX3CR1⁺ MPs in a cross-section of the tubule from the IS of control (unoperated) epididymides. Forty-eight hours after EDL (C–F), the morphology and location of MPs had been profoundly modified. CD11c⁺ and CX3CR1⁺ cells had lost

most of intraepithelial projection, and relocated into the epithelial layer. Evans blue (C, D) as well as DAPI (E, F) counterstain shows that post-EDL MPs, which contain large phagosomes, engulf cellular material, including nuclei. Occasionally, CD11c⁺ cells can be seen in the lumen (C, arrowheads). Bars = 50 μ m.

Figure 3.

Efferent duct ligation (EDL) induces marked phenotypical changes of mononuclear phagocytes (MPs) in the proximal epididymis. A flow cytometry-based analysis of mouse eMPs was performed 48 h after unilateral EDL. Cell suspensions were prepared from post-EDL and control epididymides divided in three regions representing the proximal epididymis (IS and segment 2), distal caput and corpus and cauda respectively. (A) Modulation of eMPs phenotype following EDL. Several subsets of eMPs expressing low, intermediate or high levels of CD11b, CD11c, F4/80 and CD103 were analysed. Bars

represent the mean fold change in EDL samples compared to controls, while error bars represent the standard error of the mean. (B) Flow cytometry diagrams of the most affected eMP subset (CD11b⁺ CD11c^{int} F4/80^{int}) in region 1. The density plots show events from two representative samples. CD11b⁺ cell counts were increased after EDL (highlighted in left panels, 37% vs. 18%). The analysis of the CD11b⁺ gate shows a 3-fold increase (45% vs. 15%) in cells expressing intermediate levels of CD11c and F4/80.

Figure 4.

Mononuclear phagocytes (MPs) engulf apoptotic cells in the initial segment (IS) after EDL. Forty-eight hours after unilateral EDL, numerous TUNEL-positive cells (red) are present in the epithelium of the IS (A), co-localized with actively phagocytic MPs (green, C, D). CD11c⁺ cells often contain TUNEL-positive material in their large phagosomes (D, E), indicating that IS MPs actively phagocytize apoptotic cells during the peak of epithelial apoptosis induced by EDL. Dense nuclei stained with DAPI are visible in CD11c⁺ phagocytes as early as 6 h after EDL (F, G). Bar = 50 μm (A–C), 20 μm (D, E), 5 μm (F, G).

Figure 5.

Epithelial tight junctions (TJs) are preserved after EDL in the initial segment (IS). ZO-1 immunolabelling was performed to evaluate the condition of TJs strands, which represent a key element of the blood–epididymis barrier. (A) a low-magnification image of a thick section of CD11c-EYFP mouse IS 48 h post-EDL showing abundant CD11c⁺ phagocytes (green) and the apical network of tight junctions (red) that materializes the physical separation between the luminal and interstitial environments. The high-magnification picture (B), showing ZO-1 and phagocytes as they could be seen from the lumen, reveals that TJs are relatively well preserved after EDL, despite the presence of breaches in the network (arrows). Bars = 100 μ m (A) and 50 μ m (B).

Figure 6.

Initial segment (IS) phagocytes go back to their initial appearance 4–6 days after EDL. Fluorescence microscopy images of MPs from the IS of CD11c-EYFP (A, C) and CX3CR1-GFP (B) mice harvested 4 days after unilateral EDL. Despite the non-reversible absence of luminal testicular factors, CD11c⁺ and CX3CR1⁺ MPs relocate towards the basement membrane and extend intraepithelial dendrites after the end of the EDL-induced wave of epithelial apoptosis. l, lumen.