Neutrophils and neutrophil extracellular traps enhance venous thrombosis in mice bearing human pancreatic tumors

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 doi:10.3324/haematol.2019.217083

Received: January 18, 2019.

Accepted: April 24, 2019.

Pre-published: May 2, 2019.

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Online Supplementary material

Western blot analysis for thrombus samples

Frozen thrombi were manually disrupted in radioimmunoprecipitation buffer (Thermo Fisher Scientific) and incubated on ice for 30 minutes. Lysates were cleared by centrifugation at 10,000 x g for 15 minutes at 4°C. Total soluble protein concentration was determined by bicinchoninic acid assay (Thermo Fisher Scientific). Lysates were prepared for electrophoresis by addition of SDS sample buffer (Invitrogen, Carlsbad, CA, USA) and heated to 95°C for 10 minutes. Equal amounts of total soluble protein from thrombus lysates were loaded onto 4-20% gradient SDS polyacrylamide gels (BioRad Laboratories, Hercules, CA, USA) and proteins separated electrophoretically. Proteins were transferred to polyvinylidene difluoride membranes and blocked using protein-free blocking buffer (Thermo Fisher Scientific). Membranes were probed with either anti-Ly6G (clone: 1A8, BioXCell, West Lebanon, NH, USA, 2 µg/ml), anti-β-actin (Abgent, San Diego, CA, USA, 1000-fold dilution), anti-PAD4 (Abcam, Cambridge, UK, 2000-fold dilution) anti-H3Cit (Abcam, 1 µg/ml) or anti-Histone H3 (Abcam, 0.5 µg/ml) primary antibodies. Membranes were incubated with appropriate fluorescently-labelled anti-IgG antibodies (Invitrogen, 0.1 µg/ml). Specific antigen-antibody complexes were visualized using an Odyssey imaging system (Li-Cor Biosciences, Lincoln, NE, USA). Densitometric analysis was conducted using ImageJ software (version 1.52, National Institute of Health, Bethesda, MD, USA).

Immunofluorescence

Cryosections (6 µm) were made from 8 different areas of the thrombi. Extracellular DNA was detected using TOTO-3 (Life Technologies, Budapest, Hungary). H3Cit was detected using an anti-H3Cit antibody (Abcam) and an Alexa Flour 488 goat anti-rabbit IgG (Life Technologies, Budapest, Hungary), whereas fibrin with mouse anti-human fibrin (ADI311, Sekisui, Pfungstadt, Germany) and Alexa Flour 546 goat anti-mouse IgG (Life Technologies). Areas of different fluorescent signals were quantified using Image J software[1].

Scanning electron microscopy

Thrombi were processed as described[2]. Ten images were taken of each thrombus with a SEM EVO40 (Carl Zeiss GmbH, Oberkochen, Germany). Surface occupancy of cellular

components and fibrin network was determined in 5 images at the lower magnification after dividing the images into 864 equally sized square regions of interest using Photoshop 7.0.1 CE (Adobe, San José, CA, USA). Based on morphological characteristics each region was classified as occupied by fibrin, red blood cells or their combination. Thrombus composition was then calculated as percentage of regions occupied by each component out of the total area of the image[1]. The morphometric analysis included also the manual measurement of 300 fibrin fiber diameters followed by evaluation of their distribution using scripts running under Image Processing Toolbox 10.3 of Matlab 9.5.0.944444 (R2018b) (The Mathworks, Natick, MA)[2].

Statistical procedure for comparison of pairs of distributions of randomly measured variables

Multiple number of measurements on the area occupied by a specific fluorescent signal (DNA or H3Cit) or on the morphometric characteristics of SEM images (fiber diameter or area occupied by cells) were performed in each of the experimental animals belonging to two groups – tumor (k=1) and control (k=2). These groups were composed of n_1 and n_2 number of mice, respectively. Thus, for the i-th mouse from the k-th group, we performed $m_{i,k}$ measurements of the respective signal (S), organized in the sample $\chi_{i,k} = \left\{S_1^{i,k}, S_2^{i,k}, \ldots, S_{m_{i,k}}^{i,k}\right\}$ and the goal of the statistical procedure was to identify if there was any difference in S measured in the two groups. The distributions of S in the two groups could be compared at different chosen α-quantile levels. Typically we performed comparisons for $\alpha \in \{0.25, 0.50, 0.75\}$ (bottom quartile, median, top quartile). If $\alpha_{i,k}$ was the α-quantile estimate of the i-th mouse in the k-th group, the random variable α_k (the α-quantile estimate of the k-th group) had n_k realizations calculated from $\chi_{i,k}$ for i=1,2,…,n_k. Then α_1 and α_2 could be compared by any numerical characteristics estimate $NC_{1,\alpha}$ and $NC_{2,\alpha}$ (NC could be median, mean, standard deviation, interquartile range). For example, $med_{2,\alpha} = med\left(\alpha_2\right)$ was the median estimate of the selected α-quantile estimate of the second group, whereas $IQR_{1,\alpha} = IQR\left(\alpha_1\right)$ was the interquartile range estimate of the selected α-quantile estimate of the first group. The random variables $NC_{k,\alpha}$ had a single realization in the described experiment. To compare the distribution of a pair of random variables (e.g. $med_{1,0.5}$ and $med_{2,0.5}$), we created

two samples each containing *M*=200 Bootstrap realizations of each variable. In the *r*-th pseudo-reality, for each mouse from the *k*-th group we generated a synthetic Bootstrap sample $\chi_{i,k}^{r,s}$ using drawing with replacement from the original sample $\chi_{i,k}$ [3]. We calculated $\alpha_k^{r,s}$ from $\chi_{i,k}^{r,s}$ and then compressed the n_k synthetic quantiles into the selected numerical characteristic $NC_{k,\alpha}^{r,s}$. Finally, for both groups we had a sample containing *M*=200 numerical characteristics of the selected α-quantile. The distributions of the two variables $NC_{1,\alpha}$ and $NC_{2,\alpha}$ (represented in the two samples) were compared using Bootstrap Kuiper test with *N*=10,000 pseudo-realities[4]. The evaluation procedure was performed with original functions – available at request – running under Matlab 9.5.0.944444 (R2018b) with Statistics and Machine Learning Toolbox v. 11.4 (The Mathworks, Natick, MA).

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