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Characterization of pulmonary emboli ex vivo by magnetic resonance imaging and ultrasound

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Abstract

Introduction: Magnetic resonance imaging (MRI) and transesophageal ultrasound (US) are promising methods for detection and characterization of central pulmonary emboli. Both methods employ different physical principles. We tested how US and MRI characterized pulmonary emboli ex vivo.

Methods: Thirty-six ex vivo pulmonary emboli, obtained during routine autopsies of patients who died of massive pulmonary embolism, were subjected to US imaging (linear vascular probe, 5.7–10 MHz) and to high resolution three-dimensional T1-weighted spin-echo MRI. In another three pulmonary thromboemboli and two tumor emboli, we compared MRI with immunohistochemistry to platelets, red blood cells and renal carcinoma cells. We also studied model clots in vitro (retracted and non-retracted red whole-blood clots, platelet aggregates and compacted and non-compacted fibrin-rich plasma clots) with MRI and US.

Results: T1-weighted MR images of pulmonary thromboemboli consistently showed dark regions that corresponded to red cell-rich regions and bright layers that corresponded to platelet aggregates, but bright signal was obtained also from viable carcinoma cells and necrotic regions in tumor emboli. US images provided less structural detail than MRI, but clot retraction or compaction increased image brightness. The correlation between US and MRI characteristics of pulmonary emboli was poor.

KEYWORDS
Ultrasound imaging; High resolution magnetic resonance imaging; Pulmonary emboli

Abbreviations: US, ultrasound; MR, magnetic resonance; MRI, magnetic resonance imaging.

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Conclusions: T1-weighted MRI of pulmonary emboli is capable of non-invasive assessment of the red cell-rich and platelet-rich components of pulmonary thromboemboli. US imaging shows increased brightness with clot retraction or compaction. Thus, both methods detect clot characteristics that influence susceptibility to thrombolytic treatment.

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Introduction

Magnetic resonance imaging (MRI) of pulmonary vasculature and transesophageal ultrasound (US) imaging of main pulmonary arteries are promising diagnostic methods for detecting and characterizing pulmonary emboli. However, they are not yet routinely used in clinical practice [1,2].

Transesophageal US detects the presence of proximal pulmonary emboli and can assess their mobility [3,4] which predicts survival after thrombolytic treatment [4]. Patients with immobile, echogenic central pulmonary thromboemboli had a significantly higher short-term mortality than those with mobile, echolucent thromboemboli [4].

In contemporary clinical practice, MR angiography is reserved for those patients with suspected pulmonary embolism, whom one does not wish to expose to ionizing radiation or radiographic contrast agents [1]. However, several clot characteristics may be assessed by MRI, including clot retraction [5–7]. Attempts have been made to link MRI characteristics of blood clots to their susceptibility to thrombolytic treatment [8,9].

Our aim was to test whether high resolution T1-weighted MRI in comparison with US imaging uncovers additional information about the structure of pulmonary emboli ex vivo.

Materials and methods

Pulmonary emboli ex vivo

Pulmonary emboli were collected within 12 hours after death during routine post-mortem examinations of 36 patients who died of massive pulmonary embolism. Our research did not interfere with the course of autopsy and the protocol has been approved by the Ethical Committee of the Republic of Slovenia at the Ministry of Health. The emboli were rinsed with phosphate buffered saline, pH 7.4, containing 0.2% sodium azide, and were subjected to prompt examination by US imaging. Afterwards, they were stored at −70 °C and thawed prior to examination by MRI.

In order to clarify whether T1-weighted MRI could detect the platelet component of pulmonary emboli, another 3 pulmonary thromboemboli from patients who died of massive pulmonary thromboembolism and 2 tumor emboli from a deceased patient with renal-cell carcinoma that invaded the inferior vena cava were collected for prompt analysis by US and MRI, followed by immunohistochemistry to platelets, red blood cells or renal carcinoma cells.

Artificial blood clots in vitro

In order to examine the ultrasonic and magnetic resonance imaging characteristics of different blood clot constituents, several types of clots were prepared in vitro at least in triplicate.

Whole-blood clots were prepared from citrated venous blood, donated by one of the authors, by adding calcium (2 mol/l CaCl2, 30 μl per ml of blood) and thrombin (Thrombin, Sigma, Germany) in a final concentration of 1 NIH unit/ml of blood. Clots were either allowed to spontaneously retract to about 35% of their original volume after they had been gently detached from the test tube walls with a needle, or were prevented from retracting by addition of the phosphodiesterase inhibitor UDCG 212 (Boehringer, Germany) in a final concentration of 20 μmol/l before induction of clotting [10].

Plasma clots were prepared from citrated, frozen human plasma, obtained from the Blood Transfusion Centre of Republic of Slovenia. Clotting was initiated by addition of calcium (2 mol/l CaCl2, 30 μl per ml of plasma) and thrombin (Thrombin, Sigma, Germany) in a final concentration of 1 NIH unit/ml of plasma. Some plasma clots were mechanically compressed to about 15% of their original volume. Platelet–fibrin clots were prepared from platelet concentrate, that contained from 800·10⁹ to 1600·10⁹ platelets/l, and was obtained from the Blood Transfusion Centre of the Republic of Slovenia. Platelet aggregation and clotting were initiated by adding calcium (2 mol/l CaCl2, 30 μl per ml of platelet concentrate), thrombin (Thrombin, Sigma, Germany) in a final concentration of 0.3 NIH units/ml, and collagen suspension (Dade Behring, Germany) in a final concentration of 1 mg/ml. The platelet concentrate was stirred gently with a metal hook, and the ensuing platelet–fibrin clot was withdrawn. In order to obtain platelet aggregates without a fibrin component, only collagen suspension at 1 mg/ml and epinephrine in a
final concentration of 50 μmol/l (Dade Behring, Germany) were added as agonists of platelet aggregation, and the platelet concentrate was rocked on a mechanical shaker until visible aggregates of about 1 mm in diameter were formed.

**Ultrasound (US) imaging**

*Ex vivo* and *in vitro* clots were put on a plastic carrier and submerged into saline for examination by linear vascular ultrasonic probes (Vascular 10–5 38 mm, HDI 3000, ATL, USA, for *ex vivo* pulmonary emboli and Vascular 10 L, Vivid 3, GE Medical System, USA, for *in vitro* blood clots). The imaging frequencies ranged from 5.7–10 MHz. Representative images through the longitudinal axis of the first 36 *ex vivo* emboli were recorded, printed on polaroids, digitalized by computer scanning and converted to 8-bit grey scale images. The subsequent 3 pulmonary thrombemboli and 2 tumor emboli and the *in vitro* clots were scanned longitudinally and transversally, and the images were saved as jpeg files that were converted to 8-bit grey scale images for analysis.

**Magnetic resonance imaging (MRI) and relaxation time measurements**

Pulmonary emboli and blood clots were examined by 3-dimensional high resolution MRI in a 2.35 T (100 MHz proton frequency) Oxford magnet equipped with Bruker’s gradient coils using TecMag’s NMR spectrometer and computer software. T1-weighted MRI was performed using the following parameters: echo time TE = 10 ms and repetition rate TR = 120 ms. Imaging field of view was 41 mm in all three spatial directions and imaging matrix was 256 (axial direction) by 128 by 128 (radial directions). Corresponding imaging resolution was therefore 160 μm per voxel in the axial direction and 320 μm per voxel in both radial directions. No signal averaging was used, so that the total imaging time was 33 min. A representative section along the longitudinal axis of each of the first 36 pulmonary emboli was used for image analysis, whereas for the subsequent *ex vivo* samples and *in vitro* clots, transverse as well as longitudinal sections were analyzed. In order to check for the effect of freezing and thawing, to which the first batch of pulmonary emboli had been subjected before MR imaging, *in vitro* clots were first scanned at room temperature, and for the second time after being frozen to −70 °C and thawed after one week.

T1 relaxation times, that are the major determinant of signal intensity in T1-weighted MR images, were measured in several types of clots by the inversion recovery spectroscopic pulse sequence. Since fibrin–platelet clots were inhomogeneous, the relaxation times of pixels representing platelet aggregates and those representing the surrounding fibrin clot were measured also by a sequence of 3D spin-echo MR images with a variable TR. The relaxation times of individual regions were reconstructed from the series of signal intensities using the MRI Analysis Calculator of the ImageJ program (Public domain program, National Institute of Health, USA).

**Immunohistochemistry**

Following US and MR imaging – completed no later than 6 h after autopsy – the 3 additional pulmonary thrombemboli and 2 tumor emboli were fixed in 10% buffered formalin, cut transversely and embedded in paraffin. Multiple 5 μm thick sections were cut from each sample. Initially, sections were stained with haematoxylin–eosin (HE) and Acid Picro-Mallory (PM) stain to get an overview of the structure of emboli and to demonstrate fibrin. Subsequently, sections were immunostained using monoclonal antibodies against components of platelets (anti β3 integrin Anti-Human CD61, DakoCytomation, Denmark) and erythrocytes (Anti-Human Glycoporphin A, DakoCytomation, Denmark) as previously described [11–13]. In renal-cell carcinoma emboli, monoclonal antibodies (Anti-Vimentin, DakoCytomation, Denmark, and Anti-Human Cytokeratin peptide 18, Sigma-Aldrich, Germany) were used to demonstrate the co-expression of vimentin and cytokeratins that are characteristic for this tumor. Immunostaining was performed on Ventana Benchmark automatic stainer, using the streptavidin–biotin peroxidase complex and diaminobenzidine tetrahydro-chloride as chromogen.

**Image analysis**

Images of representative longitudinal sections of pulmonary emboli were analyzed on 8-bit grey scale with ImageJ software (National Institute of Health, USA). In MR images, the darkest pixels within the embolus were assigned the signal intensity value 0 ($I_{min}$), and the brightest 255 ($I_{max}$). Arbitrarily, the cut-off value ($I_{co}$) between bright and dark parts of emboli was set at $I_{co}=I_{min}+0.55(I_{max}-I_{min})$, since two independent observers agreed that this was the threshold that allowed best visual discrimination between the dark and bright layers of the emboli. In US images, signal intensity was also expressed on a grey scale from 0 to 255. The images were normalized, so that the signal from the echolucent saline surrounding the clot represented 0 ($I_{min}$) and the
The co-localization of immunohistochemical platelet signal and bright areas in MR images was studied in 11 transverse sections of 3 pulmonary thromboemboli and separately in 7 sections of 2 tumor emboli. Due to the inevitable changes of shape during fixation and partial tissue fragmentation in preparation of thin slices for histology, the MR images could not be directly superimposed on the histological sections, but rather a semiquantitative approach was used. Each corresponding transverse section was divided into four quadrants giving 16 possible patterns for “positive” signal (insert in Fig. 3). Positive platelet signal was defined as pronounced platelet staining covering at least 25% of a quadrant area, and positive MRI signal was defined as high intensity signal (above the cut-off value $I_{\text{co}} = I_{\text{min}} + 0.55(I_{\text{max}} - I_{\text{min}})$), covering at least 25% of a quadrant area.

**Statistical analysis**

Correlation coefficients were calculated for overall image intensities of the US and MRI images of ex vivo emboli, for the overall proportions of bright areas in US and MR images, as well as for patterns of positive platelet immunohistochemistry and bright areas in T1-weighted MR images. Differences between mean signal intensities and their corresponding standard deviations in different types of artificial clots were assessed by Z-statistics. For statistical analysis,
Results

US and MR images of pulmonary emboli

In US images of the first 36 pulmonary emboli the average signal intensity and standard deviation was $55 \pm 21$ and the proportion of bright pixels was $0.32 \pm 0.21$. Not much structural detail was evident in individual images, but according to their overall intensity the images could be divided into "darker" and "brighter" emboli (Fig. 1a,b). In contrast, T1-weighted MRI showed a more detailed structure of emboli with clearly defined bright layers and darker areas (Fig. 1c,d). The average signal intensity of emboli in MR images was $88 \pm 31$ and the proportion of bright pixels was $0.27 \pm 0.09$. We found no correlation between mean signal intensities of individual emboli in US and MR images and only a weak inverse correlation.
between the proportions of bright pixels in individual emboli with a correlation coefficient of $-0.36$ ($p<0.05$).

**Co-localization of bright areas in T1-weighted MR images and immunohistochemical platelet staining**

The semiquantitative assessment of co-localization of bright layers in T1-weighted MR images and immunohistochemical staining for platelets in thromboemboli yielded a correlation coefficient of 0.7 ($p<0.05$) (Fig. 2). A representative MR image and immunohistochemistry to platelets and red blood cells are shown in Fig. 3.

In tumor emboli, the co-localization of bright areas in MR images and immunohistochemistry to platelets showed no correlation, because T1-weighted MRI showed bright signal also in cancerous tissue. A representative MR image and immunohistochemistry to platelets and renal-cell carcinoma components vimentin and cytokeratin are shown in Fig. 4.

**Images of artificial blood clots**

In US images of whole-blood clots, retraction approximately doubled the image intensity: from 25±13 before retraction to 52±20 after retraction ($p<0.001$) (Fig. 5a,b). Similarly, mechanical compaction of plasma clots increased the US signal intensity from 40±36, to 112±46 ($p<0.001$) (Fig. 5c,d).

The US image intensity of pure platelet aggregates did not differ from that of retracted whole-blood clots (53±19 vs. 52±20, $p=NS$).

In T1-weighted MRI, whole-blood clot retraction modestly increased image intensity by about 10%: from 46±12 to 55±16. Plasma clot compaction did not substantially alter the T1-weighted MR image intensity, either. Plasma clots were darker than whole-blood clots with an image intensity of only 28±10, presumably due to lack of paramagnetic hemoglobin and consequently longer T1 values.

In accordance with the findings in ex vivo thromboemboli, platelet aggregates in fibrin–
platelet clots in vitro appeared brighter than the surrounding fibrin structures with an image intensity of 70±12 vs. 34±20 (Fig. 6a,b,c,d). Pure platelet aggregates were brighter than the surrounding plasma by 20% (Fig. 6e). The T1 relaxation times of representative artificial clots are shown in Table 1.

Freezing and thawing increased the image intensity of retracted whole-blood clots in T1 weighted MR images by about 70% (from 55±16 to 86±15). Retraction of whole-blood clots or mechanical retraction or compaction.

Discussion

In order to test how T1-weighted MRI and US are able to characterize pulmonary emboli, we performed an ex vivo study on post-mortem pulmonary emboli and on model clots in vitro. Our principal findings are that (a) high resolution T1-weighted MRI distinguishes between platelet and red cell components of thromboemboli, (b) bright signal in T1-weighted MRI of pulmonary emboli may represent platelets but can not discriminate them from other "solid tissue", i.e., viable or necrotic renal-cell carcinoma, (c) US images of pulmonary emboli provide less structural detail than MRI and are not sensitive to the platelet component, but (d) in US images, overall brightness, i.e., echogenicity, of clots in vitro increases after retraction or compaction.

The finding that platelet aggregates display a bright signal in T1-weighted MRI is somewhat surprising since platelets – in contrast to red blood cells – do not contain paramagnetic hemo-globin that would enhance magnetic relaxation. However, in our in vitro experiments with artificial clots we found that platelet aggregates had a shorter T1 than retracted whole-blood clots and therefore appeared brighter in strongly T1-weighted MR images, where the pixel brightness is proportional to (1−exp(−TR/T1))exp(−TE/T2). The reported T1 values of solid tissue [14] correspond to the T1 values we measured in platelet aggregates, whereas whole-blood clots had longer T1 values than reported previously [6,15]. It is therefore likely that compactly packed platelets assume the properties of "solid tissue" where cellular proteins enhance magnetic relaxation, whereas red cell-rich parts of the clot retain abundant extracellular space [16] with low protein content. Consequently, the average T1 of whole-blood clots is longer than that of platelet clumps. This explanation is strengthened by the similar appearance of platelet aggregates and cancer tissue in T1-weighted MR images. Additionally, heterogeneity of the sample might have contributed to T1 shortening by magnetic susceptibility effects. In accordance with our findings, Viereck et al. found that platelet components of arterial blood clots in rabbits were distinctly the brightest part of thrombi in T1-weighted MR images [13]. Platelet-rich clots and platelet-rich areas in the clot are less susceptible to thrombolysis than platelet-poor clots and platelet-poor areas [17,18]. In clinical practice, tumor emboli are encountered much less frequently than pulmonary thromboemboli, but it is important to keep in mind that bright signal in T1-weighted MR images is not specific for platelets. Retraction did not strongly influence MRI signal intensity of T1-weighted images because increased concentration of deoxygenated hemoglobin mainly shortens T2 and has only a weak effect on T1 [5,6,9].

US imaging of pulmonary emboli showed less structural detail than MRI, partly because of lower two dimensional spatial resolution (160 μm×320 μm in MRI, compared to approximately 700×350 μm in US at 5–10 MHz) with an additional disadvantage of the larger voxel depth in US than in MRI. In addition, there is a fundamental physical difference between the two imaging modalities. In MRI the signal intensity is proportional to the density of hydrogen nuclei and is further modulated by the magnetization relaxation times T1 and T2, whereas in US imaging, the signal intensity is proportional to the reflected ultrasound energy, which is determined by the acoustic conductivity of the sample [20,21]. In our experiments US was not sensitive for detecting the platelet component of clots, presumably because homogeneous, "solid tissue-like" cell aggregates did not contain many acoustic boundaries and thus reflected little US signal. In contrast, Kwan et al. reported that high frequency 30 MHz intravascular ultrasound was able to differentiate between platelet-poor and platelet-rich plasma clots by the integrated attenuation value [17]. Retraction of whole-blood clots or mechanical

<table>
<thead>
<tr>
<th>Type of model clot</th>
<th>T1 (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retracted blood clot</td>
<td>892±39</td>
</tr>
<tr>
<td>Compacted plasma clot</td>
<td>2060±25</td>
</tr>
<tr>
<td>Platelet aggregate (measured by MR imaging)</td>
<td>602±155</td>
</tr>
</tbody>
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Table 1 Spin–lattice relaxation times (T1) of representative artificial clots

For retracted blood clots and compacted plasma clots T1 was measured by the inversion recovery spectroscopic pulse sequence and for platelet aggregates by the 3D spin-echo imaging sequence at variable TR. For each measured value the margins of error are given.

Characterization of pulmonary emboli ex vivo by magnetic resonance imaging and ultrasound

769
compaction of plasma clots strongly increased the signal intensity of US images, since echogenicity of blood clots correlates well with their red cell and fibrin content [22,23]. The brightest signal in US images was obtained from compacted plasma clots, presumably due to their high content of coarse fibrin strands. The image intensities of our ex vivo pulmonary emboli were closer to the values of retracted whole-blood clots than to those of non-retracted ones, indicating that most of the lethal pulmonary emboli were derived from retracted thrombi. It is noteworthy that in a previous study, increased echogenicity and decreased mobility of pulmonary emboli detected by transesophageal ultrasound in vivo predicted a worse outcome of thrombolytic treatment than in patients with mobile and echolucent pulmonary emboli [4]. Retracted blood clots are much less susceptible to thrombolytic treatment than non-retracted ones [5,24].

A limitation of the first part of our study was that US and MRI investigations were not performed simultaneously. However, our results were not an artifact, since the MRI signal intensity of whole-blood clots increased to a greater extent after freezing and thawing than that of platelet aggregates. This is probably due to the more uniform distribution of paramagnetic hemoglobin between the intracellular and extracellular space after red cell membrane disruption following freezing and thawing, whereas platelet membrane disruption has little effect on the relaxation properties of aggregates in view of the scant extracellular space. A more important limitation of our study is that MR and US images were obtained ex vivo which provided for better spatial resolution than can be routinely obtained in vivo. However, this obstacle may soon be overcome by technical advances of MR and US scanners.

We conclude that T1-weighted MRI of pulmonary thromboemboli is capable of non-invasive assessment of their platelet component, whereas increased echogenicity of clots in US images reflects clot retraction or compaction. Thus, both methods detect clot characteristics that are associated with susceptibility to thrombolysis, but further studies will be needed to test the predictive value of US and MR imaging for successful thrombolytic treatment of pulmonary embolism.

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References

[18] Collet JP, Montalescot G, Leuty C, Weisel JW. A structural and dynamic investigation of the facilitating effect of