Imaging of acute heart-transplant rejection using 99m-Technetium labelled oligonucleotides against interleukin-2 mRNA in rats

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Abstract

Objectives: Today, acute cardiac rejection is detected by endomyocardial biopsy, which harbours many risks. Thus, there is a necessity for less invasive methods. Since interleukin-2 (IL2) is over-expressed in acute graft rejection, we use radioactive DNA-fragments complementary to the mRNA of IL2 to detect graft rejection scintigraphically.

Methods: In a rat model of acute graft rejection, the oligonucleotide sequence complementary to the mRNA of IL2 is labelled with 99m-Technetium and injected intravenously. Scintigraphic and Geiger-counter activity of the transplants are evaluated and correlated with the current rejection classification of the International Society for Heart and Lung Transplantation (ISHLT).

Results: From the fourth postoperative day onwards, the scintigraphic images show a significant increase of radioactivity ($p < 0.05$) in the rejected organs than in the accepted grafts. While scintigraphy is not significantly correlated with the standard rejections classification of the ISHLT, there is significant correlation between the ISHLT classification and radioactivity in the Geiger-counter analysis.

Conclusions: Radioactively labelled anti-sense-oligonucleotides against mRNA of IL2 may be a promising approach for the detection of acute transplant rejection in vivo.

Keywords: Anti-sense; Cardiac; Graft; Oligonucleotide; Rejection; Transplantation

1. Introduction

Endomyocardial biopsy through a transjugular catheter is the current gold standard for detection and classification of cardiac graft rejection after transplantation. The specimens are histologically classified using the current standard of the International Society for Heart and Lung Transplantation. Due to their invasive nature, biopsies harbour certain risks, such as perforation of large vessels, myocardium and valves, cardiac arrhythmias and pneumothorax — especially in paediatric patients. As patients are usually under immunosuppressive therapy, the risk of infection is elevated [1], and there is a large variance in inter-observer reproducibility [2].

In addition, myocardial biopsy is not inerrant regarding its results [3], and in light of modern immunosuppressive agents, such as calcineurin inhibitors, the necessity of such invasive methods has already been frequently questioned.

Interleukin-2 (IL2) plays a key role in acute transplant rejection; its overexpression can therefore be correlated with the presence of acute graft rejection [4]. Owing to the important role of IL2 synthesis in rejection, current immunosuppressive agents are based upon IL2 blockage to prevent grafts from being rejected by the recipient [5]. This study aims to detect the expression of IL2 by using radioactively labelled DNA fragments complementary to the mRNA of IL2.

2. Materials and methods

2.1. DNA and 99m-Technetium

A DNA sequence complementary for the mRNA of IL2 in rats (5'-CCA-CCA-CAG-TTG-CTGGCCT-CA-3') was synthesised
by Thermo-Electron Ltd., and confirmed by the Basic Local Alignment Search Tool (BLAST)-Search (http://www.ncbi.nlm.nih.gov/BLAST/) of the National Library of Medicine. To increase stability against nucleases, we used a phosphorothioate-DNA, where one oxygen atom is replaced by a sulphuric atom. An amino-linker was inserted at the 5'-end of the oligonucleotide chain allowing a bifunctional chelate group to bind at its NH₂ part. We applied a method described by Hnatowich et al. [6] for radiolabelling the DNA.

99m-Technetium is a weak gamma emitter, with 140 keV of energy. It is the standard radioactive marker used in scintigraphic scans of the human thyroid gland and forms a complex with hydrazinonicotinamide (SHNH), which is attached to the DNA.

2.2. Conjugation of the DNA

For the DNA to be labelled with 99m-Technetium, SHNH must be bound to the DNA as a bridging molecule. The DNA is diluted at a pH of 8.5 and a temperature of 45 °C in 0.25 M ammonium carbonate at a concentration of 5 μg μl⁻¹ for nearly 30 min. Then, 6-hydrizinopyridine-3-carboxylic acid (HYNIC; 50 μg μl⁻¹), diluted in dimethylformamide with a concentration of 8.6 mg ml⁻¹, is added within 10 min. The solution is incubated for 60 min at room temperature.

2.3. Purification with high-performance liquid chromatography (HPLC)

After conjugation, the DNA–SHNH-complex has to be purified using high-performance liquid chromatography (HPLC; Nucleosil-Column 100-5 C18 4.6 mm x 250 mm). The following gradient is used with a flow of 1 ml min⁻¹: 5 min with 100% water, 15 min with 5% acetonitrile and 95% water and 40 min with 30% acetonitrile and 70% water. The DNA fraction appears as a peak after 23 min and is collected and concentrated with a vacuum vapouriser. It is diluted in 0.25 M bicarbonate, divided into portions of 10-μg DNA in 20 μl bicarbonate and stored at ~20 °C.

2.4. Labelling the DNA

DNA–SHNH (10 μg) is incubated with 100 μl of a tricine solution (7 mg ml⁻¹ water) for 20 min. Then, 50 MBq of 99m-Technetium and 5.5 μl of a stannous chloride (SnCl₂) solution (1 mg ml⁻¹ in 10 mM hydrochloric acid (HCl)) are added and incubated for 30 min. The solution is purified with HPLC using the above-mentioned settings; the solvent is removed using a vacuum vapouriser. The radioactive DNA is diluted in 0.6 ml 0.9% sodium chloride (NaCl) and purified with a 0.2-μm sterility-filter.

2.5. Animals

We used 3-month-old male and female rats of two different breeds. Rats of the Dark Agouti (DA; RT.av1) race always served as recipients. In the syngenic group, hearts of the same race (DA (RT.av1)) were transplanted, in the allogenic group Lewis (LEW (RT1.e)) served as donors. Animal studies were approved by the Ministerium für Umwelt, Naturschutz und Landwirtschaft des Landes Schleswig-Holstein (Ministry of Environment, Environmental Protection and Agriculture of Schleswig-Holstein) and the Ethics Committee of the University of Kiel, Germany. Animals received humane care in compliance with the European Convention on Animal Care. Anaesthesia was induced by ether and maintained with 267 mg kg⁻¹ intraperitoneally injected chlorohydrate. The animals were operated using a method described by Ono and Lindsey [7]. The grafts were harvested and implanted in the abdominal cavity with vascular connection to the inferior vena cava and the abdominal aorta. Sufficient vascular supply was determined by re-commencing of pulsation of the cardiac transplant.

The animals were examined on the 2nd, 4th and 7th day postoperatively. After the last scintigraphy, the animals we sacrificed to determine the biodistribution of radioactivity.

2.6. Allogenic group (n = 19) and syngenic group (n = 12)

After surgery, the animals were maintained without any further medical treatment. On day 7 postoperatively, seven additional allogenic animals were examined and sacrificed without scintigraphy. No graft rejection could be found in the syngenic animals and this was confirmed by the regular heartbeat of the implanted cardiac graft.

2.7. Immunosuppressed allogenic group (n = 8)

These allogenic animals were treated with FK506 (Tacrolimus, Fujisawa Pharmaceutical Co. Ltd.). This IL2-inhibitor was alternately injected (1 mg kg⁻¹ body-weight) in the right or left thigh muscle on a daily basis, beginning on the day of surgery. All animals of this group were examined on day 7 postoperatively.

2.8. Scintigraphy

The anaesthetised animals were fixed on the parallel-hole collimator of a conventional double-head gamma camera (ECAM, Siemens) in a supine position and an intravenous line was placed in a tail vein. The radioactive probe, diluted in 1 ml saline solution, was injected applying radioactivity between 10 and 15 MBq to each animal. After a series of dynamic images, the last images were acquired with an exposure time of 15 min, 2 h after injection.

Comparison of the organs was done by using region-of-interest (ROI) technique where the desired region is encircled with a mask and the emitted radioactive impulses within this area are counted. These ROIs were created of the same size (Fig. 1; ROI 1: graft, ROI 2: heart). A zone between the scapulae served as a neutral background (ROI 3). To work with comparable values, the relation between the radioactivity of

![Fig. 1. Sample of a scintigraphic image of the rat. The regions-of-interest (ROI) are encircled around the transplant (ROI 1), the heart (ROI 2) and a neutral background (ROI 3).](https://academic.oup.com/ejcts/article-abstract/37/5/1111/599956)
the graft and the background (graft—background relation) and the graft to the heart (graft—heart relation) is calculated.

2.9. Geiger-counter analysis

During autopsy on the 7th day, specimens of urine, blood, liver, spleen, kidney, duodenum, colon, lung, heart, bone, thyroid gland and the transplant were harvested, washed in saline and weighed. Radioactivity of each organ was measured in a Bohroch Geiger counter and adjusted to the specimen’s weight and the amount of the injected Mega- Becquerel. The graft—heart relation was calculated again. Due to the fact that the background used in the scintigraphy is actually an artificial volume, no graft—background relation could be calculated in the Geiger-counter analysis.

2.10. Histopathology

During autopsy, tissue was separated and conserved in formalin solution (10%) for histopathology. Haematoxylin–eosin (HE)-stained slices were independently examined by two different pathologists of the University Hospital of Kiel, Germany, to diagnose and classify graft rejection (Fig. 4). The current histological classification of cardiac graft rejection is the ISHLT classification [8,9]. The ISHLT is a very precise histological classification, taking several morphological details into account which would not be differentiated by a method detecting the overexpression of IL2. As, in clinical routine, the threshold for initiating extended immunosuppressive therapy is set at ISHLT-grade 2 (current standard of the Department of Cardiothoracic Surgery, University of Schleswig-Holstein, Campus Kiel, Germany), we chose to simplify the ISHLT classification as follows: the ISHLT grades 1A and 1B are combined to '1 — moderate rejection' and the grades 2, 3 and 4 to '2 — severe rejection'. ISHLT grade 0 stands for '0 — no rejection'.

2.11. Statistics

Normal distribution of the data could not be assumed. Therefore, data were described by median, quartile values and interquartile ranges (Box—Whisker Plots). Allogenic and syngenic animals were compared with a non-parametric procedure (Wilcoxon rank-sum test). On day 7, all groups were compared using a rank-based analysis of variances (Kruskal–Wallis Test). Significant differences between groups were evaluated by multiple pairwise comparisons (Dunn). A common significance level of $\alpha = 0.05$ was used in all tests. The coherence between scintigraphic and histological grade of rejection after ISHLT was evaluated by Kendall’s correlation coefficient. Sensitivity and specificity were calculated by standard ROC analysis. Some animals are excluded from final evaluation since they died during examination and could be histologically, but not scintographically, analysed. These animals were part of the syngenic 2-day group ($n = 1$), the allogenic 2-day ($n = 1$), 4-day ($n = 1$) and 7-day ($n = 1$) group. Occasionally, other animals could not be examined on their planned days of scintigraphy — these incidences are considered in the statistical analysis and the case numbers are corrected accordingly.

3. Results

3.1. Scintigraphy

Two days after surgery, the grafts in the allogenic group did not retain significantly more radioactivity than in the other group ($p = 0.083$; Fig. 2), seen in the graft—background relation. The situation is different on day 4, when the allogenic grafts contain more radioactivity than in the syngenic group ($p < 0.05$). On day 7, the grafts in the allogenic group accumulate significantly more radioactivity than in the syngenic ($p < 0.05$) and the immunosuppressed group ($p < 0.01$). This applies to both the graft—background relation (Fig. 3) and the graft—heart relation (Fig. 4). As expected, there is no significant difference in accumulation of radioactivity in the grafts between the syngenic and the immunosuppressed animals ($p > 0.05$). Comparing the scintigraphic results with the ISHLT classification, the retention of radioactivity in the grafts shows no significant correlation with the ISHLT classification, either in the graft—background relation ($p = 0.125$; Fig. 5) or in the graft—heart relation ($p = 0.11$; Fig. 6). A threshold of 2.24 in the graft—background relation has a sensitivity of 80% and a specificity of 61.1% for a rejection ISHLT 2 and higher.

3.2. Geiger-counter analysis

Seven days after surgery, there is a significant difference in radioactivity among the allogenic, syngenic and immuno-
suppressed group \((p < 0.001; \text{Fig. 7})\). Biodistribution also shows an accumulation of radioactivity in the liver and kidneys. The scintigraphic and Geiger-counter results show a correlation coefficient of 0.695 \((p < 0.05)\). The measured radioactivity in the graft correlates with the ISHLT classification \((p < 0.05; \text{Fig. 8})\). A threshold of 2.87 in the graft—heart relation has a sensitivity of 80% and a specificity of 72.2% for a rejection of ISHLT 2 and higher.

4. Discussion

4.1. Proof of acute graft rejection

Today, endomyocardial biopsy, described by Sakakibara and Konno [10], is the gold standard to detect acute transplant rejection. The histological specimens are judged using the classification of the ISHLT [9]. The extent of perivascular infiltration of lymphocytes is crucial for correct grading of graft rejection. In addition, degenerative signs such as sclerosis and fibrosis are taken into account.

While the participation of IL2-producing lymphocytes in graft rejection is described by Duquesnoy and Cramer [11], the coherence between organ rejection and the synthesis of IL2 was found by Suzuki et al. [4]. In addition, current immunosuppressive agents are based on the blockage of the IL2 synthesis in order to prevent graft rejection [5]. Due to the invasive nature and the associated risks [1] of endomyocardial biopsy, the necessity for less invasive and, therefore, less risky diagnostic tools has again been formulated by Patel and has already led to several different diagnostic approaches [12]. Scintigraphic methods that are based on the detection of cellular apoptosis via annexine V and antimyosin-antibodies, respectively [13], the proof of somatostatin-receptors [14] or major histocompatibility complex (MHC)-II receptors [15] or the detection of alteration in the carbohydrate metabolism of rejected monocytes [16] have also been investigated. Although some studies show a significant correlation among the serum concentrations of IL2, IL6, IL8 and tumour necrosis factor alpha (TNF-\(\alpha\)) and the grade of graft rejection, their concentration can also be elevated due to systemic infection or sepsis [17,18]. Other studies show that cytokines are not predictive for acute transplant rejection and, especially, not
for its severity [19]. Owing to this lack of specificity, none of these non-invasive methods was suitable to replace endomyocardial biopsies as a gold standard [20].

4.2. The use of anti-sense oligonucleotides in nuclear medicine

In 1996, prerequisites for anti-sense diagnostic tools in nuclear medicine were formulated by Hildebrand and Reske [21]. DNA probes with specific mRNA-sequences had already been used in the past to mark the c-myc oncogene [22], to prolong graft survival by blocking the intracellular adhesion molecule (ICAM) [23], for therapy of cytomegalovirus (CMV) retinitis [24] and to extend the survival time of cardiac transplants [25].

Hnatowich described a phosphorothioate modification of oligonucleotides in order to increase their stability against nucleases [26]; in 2001, Zhang et al. performed cellular uptake studies [27]. These oligonucleotides have also been used in this study. The mechanism of cellular uptake is not yet entirely elucidated, whereas a difference between in vivo and in vitro studies becomes apparent: in vitro studies reveal insufficient cellular uptake of oligonucleotides without the use of adjuvants, whereby comparable studies in vivo prove that clinically relevant amounts of oligonucleotides find their way inside the cells [28].

4.3. Anti-IL2-oligonucleotides as diagnostic tool for rejection

Due to their increased level of mRNA for IL2, the allogenic animals show a significantly higher accumulation of radioactivity than the control groups. This is seen in both the scintigraphic and the Geiger-counter analyses. The scintigraphic results do not correlate with the ISHLT rejection scale on a significant level, although a tendency is visible. By contrast, the Geiger-counter results show a significant correlation with the ISHLT. This can be explained in that scintigraphy is a two-dimensional technique where the radioactivity of a three-dimensional (3D) volume is counted; organic structures may superimpose and lead to falsely elevated results. The graft in the abdominal cavity is surrounded by intestine with low radioactivity. If further investigations with different nuclides and DNA sequences show a possible practicability of scintigraphy in the detection of acute graft rejection, a modified rejection scale adapted to scintigraphy should be created. Against the background of these restrictions and owing to the small number of animals used in the present study, the calculation of sensitivity and specificity is of limited value in this first investigation using molecular imaging techniques.

Further investigation is needed regarding more powerful gamma emitters, such as 18F, resulting in a higher spatial resolution of the images. The additional use of probes against the mRNA of other cytokines involved in transplant rejection could enhance the scintigraphic quality, too. Since IL2 is also involved in the rejection of other organ grafts, this method is not limited to the detection of cardiac rejection. For instance, there is as yet no reliable method to detect acute rejection of duodenal grafts. Since normal duodenum shows only very slight radioactive enhancement, acute rejection of a duodenal graft might easily show a significant radioactive accumulation.

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