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ORIGINAL ARTICLE

Increased serum creatine kinase is a reliable marker for acute transplanted heart rejection diagnosis in rats

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Summary

Different molecules have been studied as biochemical markers in heart transplantation. However, their utility is under discussion as results in human and animal models are controversial. In this work, lactate dehydrogenase (LDH), creatine kinase (CK) and cardiac troponin I (TnI) were studied as serologic markers of acute rejection after heterotopical heart transplantation in rats. In predictable rejection experiments, animals were divided into three groups: nonoperated (Lewis rats), control group (Lewis–Lewis isografts) and rejection group (Brown Norway–Lewis allografts). Nonpredictable rejection experiments were performed using nonconsanguineous Sprague–Dawley allografts. In predictable rejection experiments, LDH activity was similar between control and rejection groups. TnI values were heterogeneous in control and rejection groups. In contrast, the rejection group showed CK activity increased 4.5-fold compared with the control group. In addition to these predictable studies, we also presented novel nonpredictable experiments in which rats were divided into groups based on low and high CK activity. Histologic studies in these rats showed that none of those with low CK activity presented rejection signs, while all animals with high CK levels showed grade 2R rejection. These results suggest that CK might be an excellent marker for prediction of rejection in heart transplantation.

Introduction

Heart transplantation is the treatment of choice for end-stage heart failure. A major problem during the first year post-transplantation, and particularly during the first 3 months, is acute allograft rejection. At present, endomyocardial biopsy (EMB) [1] is the most reliable method of monitoring rejection. However, many problems associated with EMB include patient discomfort, small sample size and difficulties in biopsy interpretation and therapeutic decision-making [2]. Although EMB is considered a safe procedure, clinical risks cannot be ruled out [3]. For these reasons, a useful noninvasive marker for graft rejection diagnosis is of great interest. Several groups have tested biochemical markers in patients, including creatine kinase (CK) [4,5], troponin T and I [6–10] and interleukins [11], although the results are contradictory.

In addition to biochemical markers, other noninvasive methods such as heart autofluorescent spectrum analysis [12], ultrasound imaging [13] and frequency domain analysis of electrocardiogram have been studied in rats [14]. Nevertheless, disadvantages such as high cost and insufficient reliability outweigh the advantages; therefore, the routine use of these methods is difficult.

In the present work, we studied the usefulness of three biochemical markers (lactate dehydrogenase (LDH),

troponin I (TnI), and CK) to determine rejection prediction in an experimental model. The results demonstrate that serum CK levels are an excellent marker for prediction of heart rejection in rats.

Materials and methods

Animals

All animal protocols were approved by the University of Barcelona Ethics Committee and animals were handled in accordance with the European legislation on the care of experimental animals (86/609/EEC). All rats were acclimatized for 1 week prior to surgery.

The study was divided into two arms: predictable and nonpredictable rejection. In the predictable rejection experiments, rats were divided into three groups: (i) nonoperated Lewis rats (NO); (ii) control group (CG) Lewis–Lewis isografts; and (iii) rejection group (RG) Brown-Norway–Lewis allografts. All donor animals weighed 200–225 g and recipients weighed 225–250 g at the time of surgery. In these experiments, eight rats were studied in each group and all determinations were performed in triplicate. In the nonpredictable rejection experiments, nonconsanguineous 225–250 g Sprague–Dawley rats were used both as donors and recipients. Twelve rats were studied and all determinations were performed in triplicate.

Heart transplantation

Heterotopical heart transplantation was performed basically as previously described [15]. Briefly, donor animals were anesthetized with isoflurane and bilateral thoracotomy was performed. All vena cavae and right atria were ligated. Ascending aorta and pulmonary artery were cut and the heart extracted and stored in cold cardioplegic solution. Recipient animals were anesthetized as described previously, a long mid-abdominal incision was made and the abdominal vessels under the renal vessels were separated. Donor pulmonary artery and ascending aorta were anastomosed to recipient inferior vena cava and abdominal aorta, respectively, with an 8/0 prolene suture. After complete irrigation, the donor heart was checked for normal beating and the abdomen was closed in a double layer with 3/0 suture.

Postsurgical follow-up, killing, and sample extraction

All animals were monitored daily and their heart function checked by abdominal palpation. In the predictable rejection experiments, animals were kept alive for 5 days postsurgery, and then anesthetized for sample collection and killed. Blood was extracted from the inferior vena cava and serum was obtained by centrifugation. The transplanted heart was excised and immediately frozen. Hearts were sliced in a JungCM1800 cryostat (Leica Microsystems, Wetzlar, Germany) and stained with hematoxylin–eosin. Rejection grades were determined according to the new ISHLT standard (grades 0R–3R) [16]. In the nonpredictable rejection experiments, approximately 30 μl of blood was extracted daily from the tail vein and CK activity was determined. Animals showing CK activity levels under 80 U/l for 15 days were processed on that day as described previously and samples were classified in the low CK activity group. Animals with CK activity over 80 U/l, reached between days 11 (two animals) and 13 (two animals) postsurgery, were killed on that day and samples were obtained and classified as high CK activity group.

Biomarker determination

Lactate dehydrogenase and CK were measured spectrophotometrically according to Bergmeyer *et al.* [17]. Serum TnI was determined using the AccuTnI kit in an Access instrument (Beckman Coulter, Fullerton, CA, USA). To calibrate the kit for rat TnI, cDNA was amplified from a heart library and cloned into pGEX-4T-1 expression plasmid. The expressed protein was purified according to plasmid instructions and quantified in an SDS-PAGE gel using bovine serum albumin as standard. Increasing amounts of pure protein were quantified using the AccuTnI kit.

Statistical analysis

Statistical analysis was performed by commercially available software (Statgraphics plus 5.0, StatPoint Inc., Herndon, VA, USA). Differences between groups were tested by Mann–Whitney *U*-test. For all statistical analyses, $P < 0.05$ was considered significant.

Results

Predictable rejection experiments

All grafts from both control and rejection groups had palpable contractions when checked prior to extraction and all showed good to excellent function. Hematoxylin–eosin staining of slices showed that all hearts from CG had no rejection, while all the RG had moderate to severe rejection, grades 2R or 3R according to the new ISHLT standard (Table 1 and Fig. 2b and c).

Serum LDH activity in the different groups of rats is depicted in Fig. 1. As shown, LDH activity was increased 10.8-fold in the control group (128 ± 35 U/l) versus nonoperated rats (28.6 ± 12 U/l). However, no difference was detected between control and rejection groups.

Creatine kinase activity was increased only 3.7-fold in the control group (42 ± 2.1 U/l) compared with the

Table 1. Serum cardiac troponin I in nonoperated (NO), control (CG) and rejection (RG) rat groups.

Nonoperated		Control group		Rejection group	
Rat no.	ng/ml	Rat no.	ng/ml	Rat no.	ng/ml
NO1	nd	CG1	0.25	RG1^{2R}	3.69
NO2	0.03	CG2	0.96	RG2^{2R}	0.40
NO3	nd	CG3	0.12	RG3^{3R}	2.38
NO4	0.06	CG4	0.16	RG4^{3R}	20.04
NO5	nd	CG5	0.35	RG5^{2R}	8.88
NO6	0.02	CG6	0.40	RG6^{2R}	5.38
NO7	nd	CG7	16.25	RG7^{2R}	12.91
NO8	nd	CG8	20.38	RG8^{3R}	11.91

Values are means of three determinations (ng/ml).
^{2R,3R}Rejection grade. nd, nondetectable.

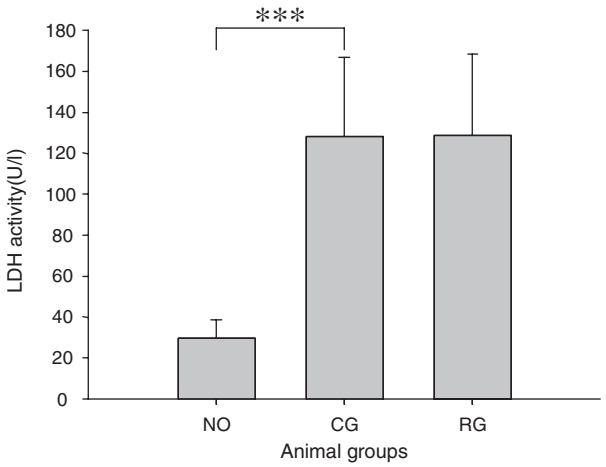

Figure 1 Lactate dehydrogenase activity levels in predictable rejection experiments: nonoperated rats (NO, eight rats), control group (CG, eight rats) and rejection group (RG, eight rats)). Values are means ± standard deviation. ***$P < 0.001$ between NO and CG.

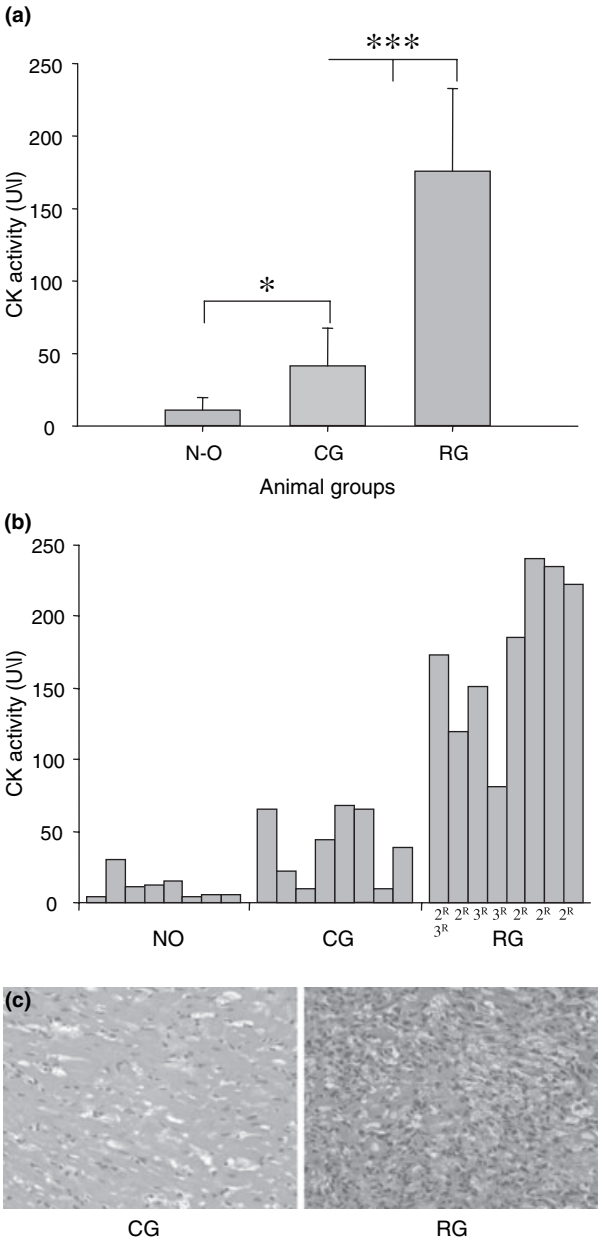

Figure 2 Creatine kinase activity levels in predictable rejection experiments. (a) Creatine kinase activity in nonoperated rats (NO, eight rats), control group (CG, eight rats) and rejection group (RG, eight rats). Values are means ± standard deviation. *$P < 0.05$ between NO and CG, ***$P < 0.001$ between CG and RG. (b) Creatine kinase levels in all rats are depicted individually and rejection grade is indicated for all animals of the RG. (c) Histological analysis of representative hearts from control and rejection groups.

nonoperated group (11 ± 1 U/l) (Fig. 2a). Nevertheless, the rejection group showed a high increase in CK activity (175.8 ± 57 U/l) (Fig. 2a), which corresponded to increases of 16-fold and fourfold compared with nonoperated and control groups, respectively. It is noteworthy that all RG animals tested showed CK activities greater than those of CG, as shown in Fig. 2B. Nevertheless, in these predictable rejection experiments, no significant difference was observed between 2R and 3R rejection grades (2R: 191 ± 49 U/l; 3R: 150.2 ± 71 U/l).

Troponin I levels in the three rat groups are shown in Table 1. In the nonoperated group, all animals showed blood TnI levels below 0.06 ng/ml and in five of the eight rats analyzed TnI was not detectable. In the control group, TnI levels were significantly higher, in six of the eight animals tested, than in nonoperated groups, although the values remained very low (<1 ng/ml). Nevertheless, in two animals, TnI levels were high (16–20 ng/ml). TnI analyses of the rejection group showed that seven of the eight rats presented high TnI levels (2.4–13 ng/ml), although one rat presented low TnI

levels (0.40 ng/ml). When all animals from both control and rejection groups were considered, no significant differences were observed between GC (4.9 ± 8 ng/ml) and RG (8.2 ± 6.5 ng/ml) or between 2R and 3R rejection grades in the RG group (2R: 6.3 ± 4.8 ng/ml; 3R: 11.4 ± 8.8 ng/ml) (Table 1).

Nonpredictable rejection experiments

Random rejection experiments were performed using nonconsanguineous Sprague–Dawley rats both as donors and recipients to demonstrate the validity of CK activity as a marker of heart rejection. CK activity was measured daily post-transplant. This activity remained constant during the first week postsurgery and all values were between 30 and 40 U/l (Fig. 3a), similar to those obtained in the control group in the abovedescribed predictable rejection experiments.

According to their CK activity, rats were divided into two groups: low CK activity ($n = 8$) with CK values under 80 U/l before 15 days after transplantation, and high CK activity ($n = 4$) with values over 80 U/l, which were fourfold those of the low CK activity group (Fig. 3b). Hearts were harvested, sliced and stained with hematoxylin–eosin. None of the rats in the low CK activity group showed signs of rejection, whereas all rats in the high CK activity group showed rejection grade 2R (Fig. 3c).

Discussion

Since EMB was first introduced, it has been used as the gold standard for rejection diagnosis [1]. However, the drawbacks of EMB, such as patient discomfort, clinical risks or elevated economic cost, an alternative, noninvasive method of rejection diagnosis which would improve patient follow-up and the long-term success rate of heart transplantation. Unlike EMB, serum protein levels and enzyme activity determination constitute a rapid, reproducible method which requires small amounts of sample.

Lactate dehydrogenase is a biochemical marker used in the diagnosis of heart injury, diagnosis, although its role as a rejection marker has been scarcely studied. Our results showed no difference between nonrejected and rejected groups and, consequently, LDH was not further studied and ruled out as a rejection marker.

The results of CK activity presented here, as tested in our experimental model, showed a significant difference between nonoperated and control groups. This increase in activity can be attributed to surgery injury. However,

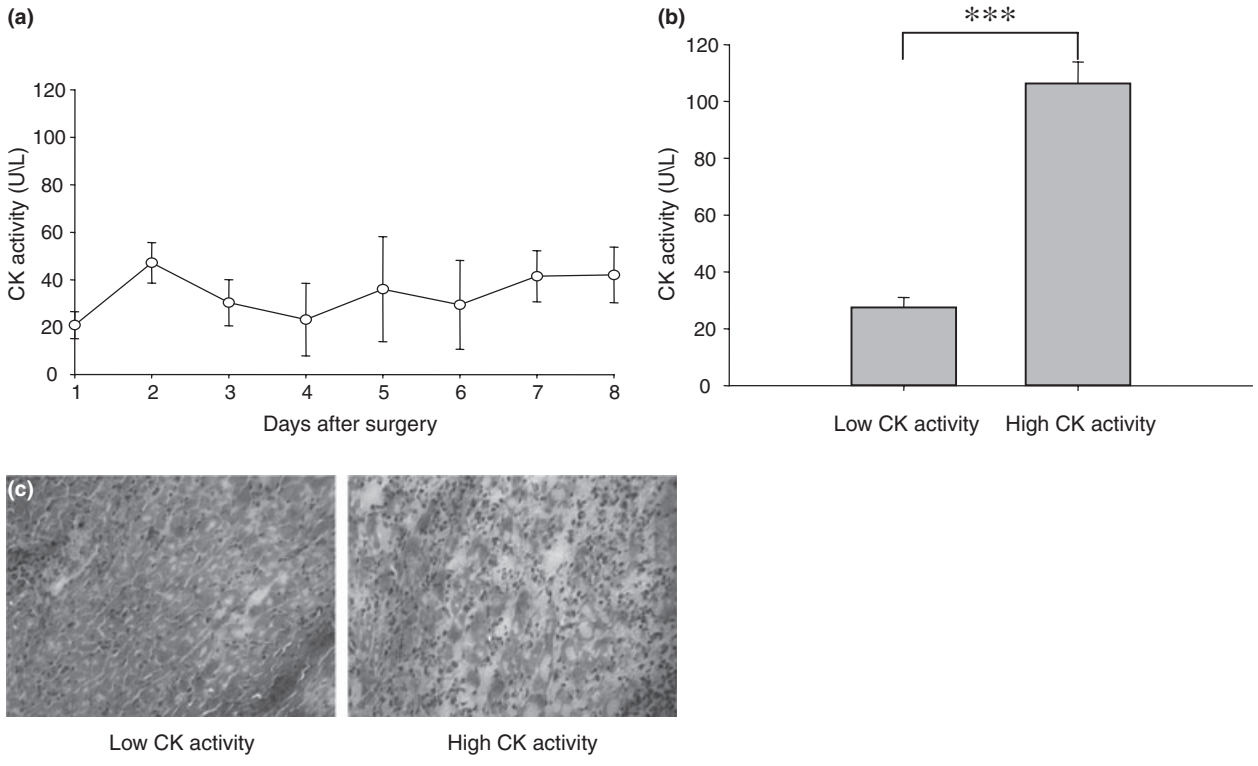

Figure 3 Creatine kinase (CK) activity levels in nonpredictable rejection experiments. (a) Activity levels during the first 8 days postsurgery. Values are means ± standard deviation. (b) CK levels for the low (eight rats) and high (four rats) CK activity groups. ***$P < 0.001$ between low and high CK activity groups. (c) Histological analysis of representative hearts from low CK activity and high CK activity groups.

when control and rejection groups were compared, CK activity was increased 4.5-fold, well over the increase between the former two groups; thus, this increase in CK activity levels can be attributed to heart rejection. Moreover, it is noteworthy that when animals of the rejection group were analyzed individually, all values were above those of controls, which suggests that CK could be used as a marker. Furthermore, no false positives were detected. In addition, no significant CK level differences were detected between rats with 2R (191 ± 49 U/l) and 3R rejection grades (151 ± 71.0 U/l). These results concur with those obtained by Walpoth *et al.* [18], where no difference was observed in CK activity levels between moderate and severe rejection.

A useful biochemical marker should be able to diagnose rejection episodes without the need for EMB. In this case, high levels of marker in serum should correspond to an acute rejection episode. This is particularly important in small animal models where EMB is not an option for following heterotopical transplantation, as it is in patients. To confirm whether this is the case for CK activity as a rejection marker, we designed a novel approach, the nonpredictable rejection experiments, using nonconsanguineous rats of the same strain as both donors and recipients. In this model, transplanted heart could present rejection episodes between 10 and 15 days, or no rejection in at least 30 days. CK activity was followed on a daily basis. Animals were classified according to their CK activity levels instead of rejection grade, and rejection was later determined histologically. No animals in the low CK activity group showed signs of rejection. In contrast, all animals in the high CK activity group showed grade 2R rejection. Moreover, no false positives or negatives were detected. These results confirm the validity of serologic CK activity as a rejection marker in our model.

Creatine kinase is widely used in heart damage assessment in many experimental animal models such as ischemic preconditioning [19] and cold cardioplegic arrest [20]. Its activity evaluation is rapid and reproducible, and small amounts of sample are required. In patients, it has been proposed that CK, particularly the CK-MB isozyme, is useful only in certain cases as a heart rejection marker [4,21]. Studies with rats could present some limitations for clinical application owing to the different characteristics between humans and animals. Nevertheless, the novel nonpredictable experiments presented here are those which most closely resemble the human situation. In addition, the fact that CK allows for determination of moderate grade 2R rejection may permit the study of different pharmacologic treatments in rats useful for later use in humans.

Troponins have been amply studied as biochemical markers in infarcted patients. Moreover, both troponin T (TnT) and TnI, particularly the former, have also been studied as possible markers of heart rejection. In humans, data are, at present, contradictory. Some authors [8,22,23] have proposed that TnT could be used in patients for heart rejection diagnosis, albeit with some restrictions. Other works suggest that TnT could be a good marker to assure rejection if combined with EMB [24,7,10]. In contrast, other authors suggest that TnT cannot be used as a rejection marker [6,5,9,25]. Unlike in humans, Walpoth *et al.* [18] detected some correlation between TnT levels and rejection grade in rats; nevertheless, because of the wide deviation among values, the results are not conclusive.

TnI has been studied less than TnT as a rejection marker. Thus, Forni *et al.* [26] were unable to demonstrate a correlation between human TnI levels and heart rejection while, more recently, in pediatric transplantation, it has been suggested that TnI, though specific, is also an insensitive marker and can only be used in some cases [27].

In nonoperated rats, blood TnI levels were very low or not detectable. In the control group, 75% of rats showed slightly higher TnI levels (between 0.2 and 0.9 ng/ml) although the two remaining rats showed TnI levels well above the nonoperated group levels (16–20 ng/ml). In those two animals, no surgical problems arose; moreover, as the histological analysis showed no myocardial damage, these animals were included in the study. Moreover, in the rejection group, 88% of rats showed high TnI levels, but the rest showed low TnI levels similar to those of the control group and, therefore, these data cast doubt on the validity of TnI as a rejection marker, particularly when compared with CK for which no false positives or negatives were detected. Although the use of TnI as a rejection marker could not be ruled out in other experimental models, the false positives in the control group and the false negatives in the rejection group suggest that TnI is insensitive and nonspecific as a rejection marker in rats.

In conclusion, this work demonstrates that CK is a good biochemical marker for rejection in heart-transplanted rats, as all animals with rejection presented high CK levels, >80 U/l. It is noteworthy that measurement of CK levels permits detection of moderate or severe rejection grades in transplanted hearts and, when determined daily, moderate rejection can be detected promptly. In addition, when CK activity was used for animal post-transplantation follow-up, it also proved to be a good diagnostic tool for acute heart rejection prediction. Furthermore, CK level determination is a noninvasive, low-cost, sensitive, easy-to-perform method. Although rats and humans could respond differently to heart rejection, we believe that the nonpredictable rejection experiments presented here constitute a good approach to studying possible therapies to reduce the heart rejection rate in animals, which could be extended to humans.

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