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New Biomarkers in Screening Anthracycline-Induced Cardiotoxicity Only with Peripheral Blood Sampling

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Abstract

Because oxidative stress after administration of doxorubicin was identified as playing a central role in cardiac dysfunction, we hypothesized that the expression (or overexpression) of TLR2 and TLR4 contributes to the pathogenesis of doxorubicin-induced cardiac dysfunction. Toll-like receptors (TLRs) are members of the interleukin-1 receptor family (IL1) and are involved in the ability to react to the molecular trigger associated with pathogenic microorganisms. Recent studies have shown that TLR receptors are activated by endogenous signals, such as heat shock proteins and oxidative stress, which can contribute to congestive heart failure. Until recently, the best detection method for cardiotoxicity induced by anthracyclines was myocardial biopsy. Other early screening and early diagnosis methods (biomarkers—cardiac troponins and natriuretic peptide) have not yet proven their efficacy. Our proposed method is a new, revolutionary one that does not imply any kind of physical (and psychic) aggression on the patient: the targeted genetic (TLR2/TLR4) analysis of the human peripheral blood (which is a minimally invasive procedure).

Keywords: anthracycline, cardiotoxicity, doxorubicin, Toll-like receptor, biomarker

1. Introduction

Immunotherapy for cancer has been and still is a subject of intense research since the nineteenth century when Coley noticed that bacterial components can contribute to cancer regression and can cause an immune antitumor immune response.

Permanent cardiotoxic effects of conventional cytostatic therapy underscore the need for early diagnostic methods with great sensitivity to allow early detection of signs of cardiac dysfunction, the consequence of the cardiotoxic effects of cytostatic medication. The specificity of any test should allow for a precise risk/benefit analysis, a balance between the probability of cardiac dysfunction due to high drug doses and the consequences of stopping antitumor therapy.

Several Toll-like receptors (TLRs) are expressed in cardiomyocytes, including TLRs 2 and 4. Through these TLRs, cardiomyocytes respond to endogenous or exogenous signals that can influence pathophysiological responses to dilated cardiomyopathy. Expression or activation of both TLR2 and TLR4 are overregulated in experimental and in vivo (hypertensive and/or clinical heart failure patients) models. Therefore, inhibition of TLR signaling may be of great therapeutic benefit for dilatation cardiomyopathy (CMD), especially in the treatment with doxorubicin.

2. Serum markers: cardiac troponins and natriuretic peptide

For cardiovascular disorders, including doxorubicin-induced heart failure, an increased number of candidate markers were studied to be useful in detecting myocardial injuries. These include cardiac troponins and natriuretic peptides.

However, most of the results have highlighted the fact that some of the patients have detectable troponin, suggesting that this may be considered as a prognostic marker for doxorubicin-induced cardiotoxicity.

Cardiac troponins are complex joints of thin filaments that are involved in the regulation of actin-myosin coupling in the cardiac muscle and consist of three subunits: troponins T, C, and I [1–4].

Cardiac troponins T and I are both highly sensitivity and have specificity for myocardial injuries [5]. Both markers are used as prognostic and diagnostic tools in acute coronary syndrome [6]. Cardiac troponins have also been studied from the perspective of use as markers for anthracycline-induced cardiotoxicity, with bivalent results [7].

These contradictory results can be determined by many factors, such as heterogeneous population studies, variable cumulative doses of anthracycline, and different study protocols with different working methods.

The utility of troponins was mainly demonstrated after administration of antineoplastic and β-sympathomimetic drugs, although the routine use of these markers in monitoring in patients receiving anthracycline therapy is far from being solved.

3. Toll-like receptor and cardiotoxicity

Toll-like receptors (TLRs) are members of the interleukin-1 receptor family (IL1) and are involved in the ability to react to the molecular trigger associated with pathogenic micro-organisms. Recent studies have shown that TLRs are activated by endogenous signals, such as heat shock proteins and oxidative stress, which can contribute to congestive heart failure.

Oxidative stress is one of the major doxorubicin (Dox)-induced cardiac dysfunctions. Thus, we hypothesized that TLRs contribute to the pathogenesis of doxorubicin-induced cardiac dysfunction.

Toll-like receptors (TLRs) recognize pathogens associated with molecular patterns such as lipopolysaccharides, peptidoglycan, bacterial lipoproteins, and oligonucleotides during inflammatory response.

TLRs have interleukin-1 (IL1)-like response pathways as an intracellular signaling means leading to the nuclear localization of the kb/Rel-type nuclear transcription factor (NF). Moreover, TLRs are expressed in various organs, such as the lung, brain, kidneys, heart, etc.

Recent studies have suggested that the "activated myocardium" through mediated TLR signaling pathways in response to exogenous ligands induces myocardial dysfunction.

Other studies analyzed by us for scientific documentation have also demonstrated that there are other signaling-mediated TLR pathways that are activated by endogenous signals such as heat shock protein and oxidative stress in cardiomyocytes isolated from ventricular level.

We must also keep in mind that it has recently been shown that TLR2 plays an important role in ventricular remodeling after myocardial infarction and also with cardiac specificity (TLR4 compared to it does not have a system/organ specificity).

Doxorubicin (Dox) is an effective antitumor antibiotic in the anthracycline class. However, doxorubicin also induces cardiomyopathy leading to congestive heart failure, thus often limiting its clinical use.

Doxorubicin-induced cardiomyopathy is mainly caused by increased cardiac oxidant production.

It has also been reported that treatment with doxorubicin causes the release of cytochrome C and results in the activation of caspase 3 and cellular apoptosis.

These studies also indicate that free radicals play an important role in doxorubicin-induced cardiotoxicity.

Because oxidative stress after administration of doxorubicin was identified as playing a central role in cardiac dysfunction, we hypothesized that the expression (or overexpression) of TLR2 and TLR4 contributes to the pathogenesis of doxorubicin-induced cardiac dysfunction.

Successful activation and maturation of specific tumoral immune cells are known to be mediated by bacterial endotoxin, which activates Toll-like receptor 4 (TLR4). TLR4 is expressed

on a variety of immune and tumor cell types, but its activation may have opposite effects. While activation of TLR4 may promote antitumor immunity, it can also cause excessive tumor growth and immunosuppression.

However, TLR4 binding to endotoxin, as well as endogenous ligands, is a notable contribution to the outcome of treatment of various cancers, such as radiation or chemotherapy. Further research into the role and mechanisms of TLR4 activation in cancer may provide new antitumor adjuvants as well as TLR4 inhibitors that could prevent inflammation-induced carcinogenesis or the cardiotoxic effect of anthracyclines in treatment.

The immune system plays an important role not only in defending against microbial infection but also in controlling and monitoring malignant tumors.

Immune cells scan the tissues with the objective of eliminating newly formed malignant cells before transforming into fully formed tumors. Malignant cells develop complicated mechanisms that allow them to inhibit immune cells by secreting specific cytokines that create an immunosuppressive medium [1]. Tumors can even directly kill the tumor-lymphocyte infiltration, which are CD95 sensitive, by expressing CD95L (Fas ligand) [2].

So, inborn immunity is the first line of defense against microbial infection. The innate immune system cells can recognize the pathogen, and the appropriate immune response is triggered by Toll-like receptors (TLRs), undoubtedly the most important sets of immune-derived vertebrate receptors. TLRs recognize different molecules of microbial origin, called associated pathogenic molecular models. TLRs are located on the cell surface (TLR1, 2, 4, 5, 6) or endosomal compartments (TLR3, 7, 8, 9) with the main role to protect the body from infection. After recognizing these ligands, the TLRs dimerize and trigger a cytoplasmic signaling pathway that leads to the activation of several nuclear factors (e.g., NFκB, IRF) responsible for transcription of immune system genes [3].

Signaling of TLR receptors in immune system cells is critical for regulating innate and adaptive immune response, such as for antigen maturation and presentation as well as for CD8 + and T-cell cytotoxicity, all of which are important factors in antitumor immunity [4]. On the other hand, TLR stimulation may also lead to the proliferation of improved T-cell regulatory and suppressor-like functions in tumor development [5–7].

TLR expression is not limited to immune cells, and indeed many tumor cells have been determined by expression of TLRs, signaling by which they can stimulate tumor growth and immune system evasion [8, 9]. On the other hand, TLR signaling in tumor cells has also been demonstrated to reduce the proliferative capacity of tumor cells [10]. We will focus on reports on TLR4 signaling and its involvement in cancer development and progression.

3.1. Toll-like receptor 4 in health and sickness

TLRs are Toll homologs, insect receptors, which are involved in the determination of dorsoventral polarity during embryogenesis, as well as in the immune response against fungal infections [11, 12]. The first to be found in the human race was TLR4. Endotoxin (i.e., lipopolysaccharides), an external membrane component of Gram-negative bacteria, which is composed of a preserved amphipathic lipid and other variable polysaccharides, is also recognized.

The TLR4 activation mechanism is quite complex and (unlike other TLRs) involves several auxiliary proteins (LBP, CD14) and a coreceptor (MD-2) [3] (**Figure 1**). This is actually MD-2 and not TLR4 that recognizes and binds directly to endotoxin [13, 14]. MD-2 is a soluble protein with a large hydrophobic pocket that represents the binding site for the lipid A lipid chains. Lipid A is usually composed of six acyl chains, but only five of them are bound in the hydrophobic pocket of MD-2.

The sixth acyl chain leaves the pocket and interacts with hydrophobic residues on the TLR4. These interactions are crucial for MD-2/TLR4 heterodimerization and are therefore a prerequisite for TLR4 activation, a cascade signaling [15, 16].

3.1.1. Recommendations for the evaluation of cardiac biomarkers: BNP and cTnI

Recommended method: microparticle enzyme immunoassay (MEIA), quantitative determination of troponin I-cTnI in serum or plasma (troponin I cut-off value <0.04 ng/ml), and quantitative determination of BNP in plasma (BNP-100 pg/mL cut-off value).

Sample collection: from each patient, take 2 ml of fresh blood for each sample collected in plastic tubes; subsequently centrifuge and store plasma/serum samples under optimum conditions at −20°C; and the maximum limit of 2 months is not exceeded.

The number of samples taken from each patient should be two, with a certain periodicity: initially (before initiation of treatment), after the first three cycles of doxorubicin-anthracyclines, and 3 months after initiation of treatment or from inclusion in study.
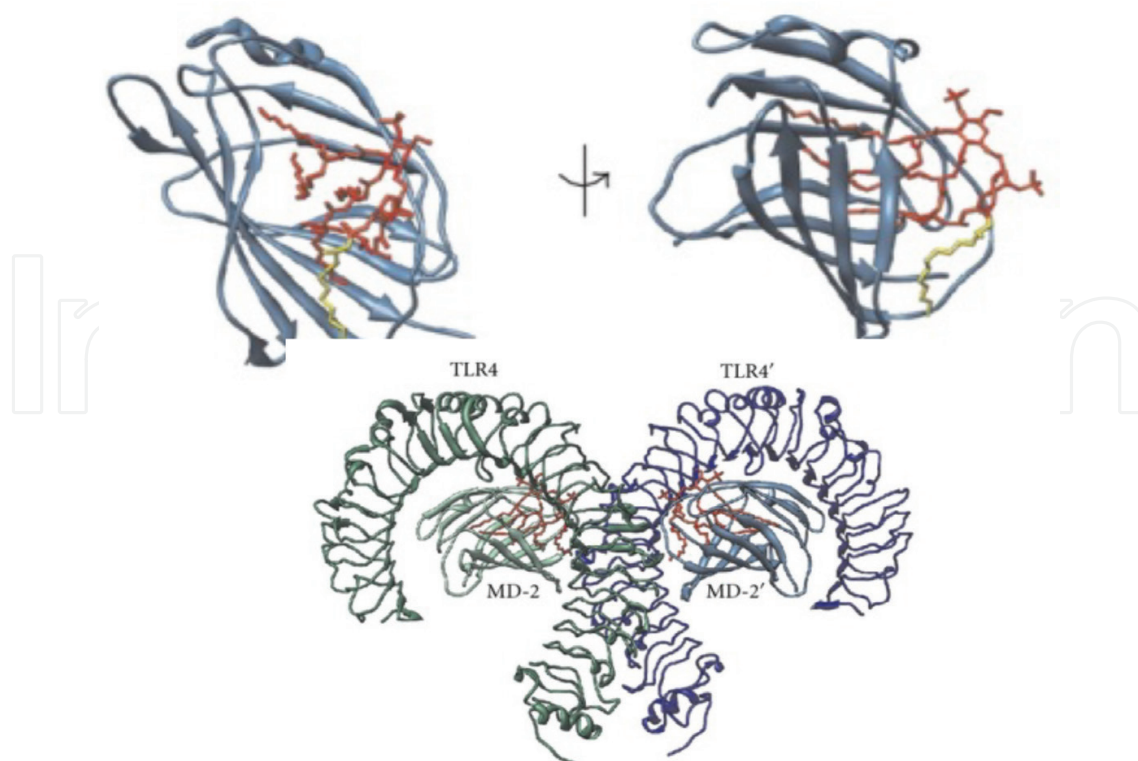

Figure 1. TLR4 spatial representation.

3.1.2. Genetic study for TLR2 and TLR4

As previously mentioned, Toll-like receptors (TLRs) are members of the interleukin-1 receptor family (IL1) and are involved in the ability to react to the molecular trigger associated with pathogenic microorganisms. Recent studies have shown that TLR receptors are activated by endogenous signals, such as heat shock proteins and oxidative stress, which can contribute to congestive heart failure.

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3.1.3. TLR2 and TLR4 genetic study methods

For sampling we recommend the use of Tempus ™ Blood RNA Tube tubes (4,342,792, Applied Biosystems®—**Figures 2–4**).

Figure 2. Tempus™ blood RNA tubes.

A total of 12 ml (9 mL sample + 3 mL PBS)

3 mL PBS

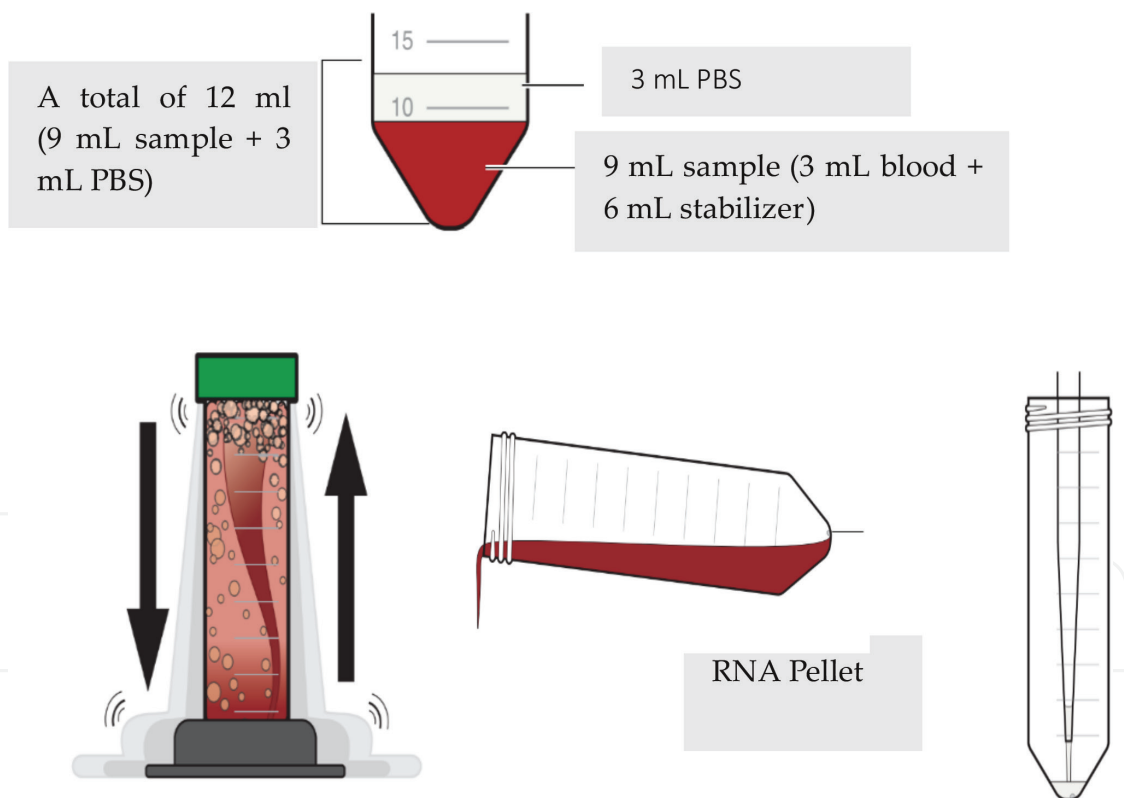9 mL sample (3 mL blood + 6 mL stabilizer)

RNA Pellet

Figure 3. Graphic representation of the RNA isolation procedure.

The tubes contain 6 ml Stabilizing Reagent.

Each tube will harvest 3 ml of peripheral venous blood from patients.

After harvest, the blood is mixed with the cell lysate stabilizing solution at the same time. The stabilizing agent inside the tube contains inhibitors of RNase while maintaining stable gene expression for more than 7 days at 4°C.
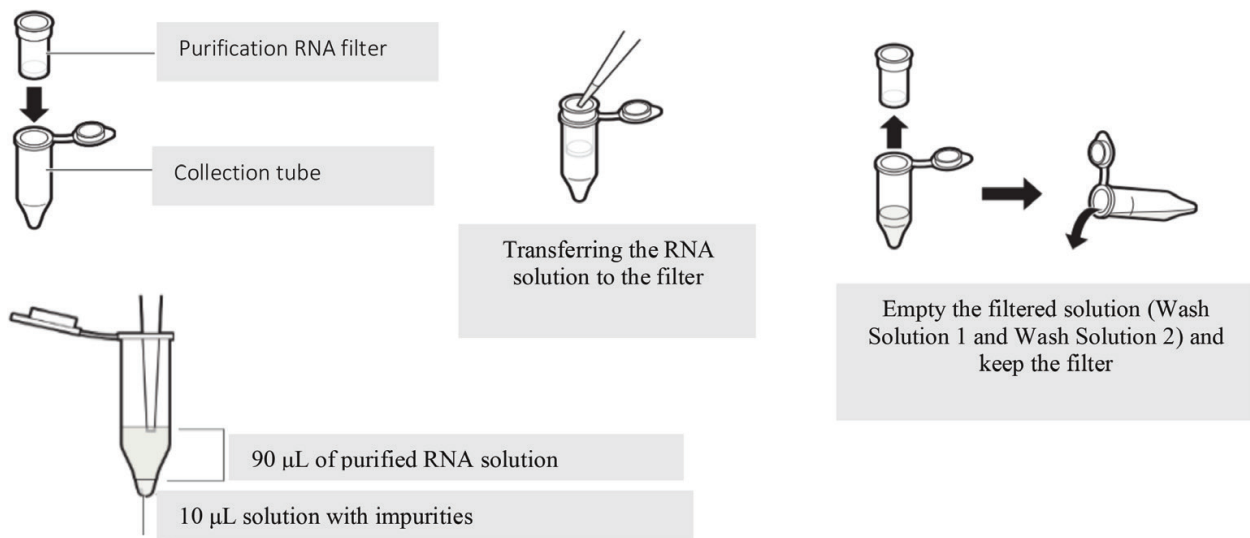
Purification RNA filter

Collection tube

Transferring the RNA
solution to the filter

Empty the filtered solution (Wash
Solution 1 and Wash Solution 2) and
keep the filter

90 μL of purified RNA solution

10 μL solution with impurities

Figure 4. Graphic representation of the RNA isolation procedure.

The stabilizing solution allows the RNA to precipitate by maintaining the DNA and proteins in the solution.

After harvesting, samples should be kept at 4°C until RNA is isolated. Isolation of RNA will take place within 5 days of harvesting.

The RNA isolation procedure includes:

The contents of the Tempus ™ Blood RNA Tube tubes were transferred to 50 mL tubes.

3 mL of PBS will be added.

Stir for 30 seconds after which the samples are vortexed at 3000 g for 30 minutes.

After centrifugation, the supernatant is discarded, and the tube is left in place for 1–2 minutes on an absorbent material.

The RNA pellet is resuspended in 400 μL of Purification Resuspension Solution and vortexed briefly; after resuspension the RNA will be purified.

3.1.4. Obtaining complementary DNA (cDNA)

For the reverse transcription reaction, the High Capacity cDNA Reverse Transcription Kits (4,368,814, Applied Biosystems) kit will be used.

The procedure for obtaining cDNA is:

Two master RT mixes according to **Table 1** will be prepared.

Use 10 μL of 2X RT and 10 μL of RNA for a reaction, to obtain the RNA solution; the RNA samples should be diluted so that in the final 100 ng/5 μL RNA solution 400 ng RNA will be used for a 20 μL reaction volume.

The amplification reaction will be performed using the 2720 Thermal Cycler (Applied Biosystems) according to the specified program (**Table 2**).

Component	Quantity (μL)
10✕ RT Buffer	2.0
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H$_2$O	3.2
Total volume/reaction	10.0

Table 1. Preparation of 2 × RT master mix with inhibitor/reaction RNase.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

Table 2. The reverse transcription program.

TLR 2	Sequence	Bp	Tm	GC%	Product length
Forward primer	**GGCATGTGCTGTGCTCTGTT**	20	61.52	55.00	125
Reverse primer	**GCTTTCCTGGGCTTCCTTTT**	20	58.66	50.00	
TLR 4	**Sequence**	Bp	Tm	GC%	Product length
Forward primer	**TTGAGCAGGTCTAGGGTGATTGAAC**	25	62.54	48.00	143
Reverse primer	**ATGCGGACACACACACTTTCAAATA**	25	61.72	40.00	
GAPDH	**Sequence**	Bp	Tm	GC%	Product length
Forward primer	**GCACCGTCAAGGCTGAGAAC**	20	61.57	60.00	138
Reverse primer	**TGGTGAAGACGCCAGTGGA**	19	61.14	57.89	

Table 3. The sequence of used amplicons.

3.1.5. Determination of relative gene expression for TLR2 and TLR4 from isolated RNA samples

For qRT-PCR amplification, the LightCycler 480 SYBR Green I Master kit, which is optimized for the LightCycler 480 Thermocycler, is used. The GAPDH was chosen as the reference gene. The sequence of amplimers used for the experiments is shown in the following table (TLR2 and TLR4 expression in peripheral blood mononuclear cells of patients with chronic cystic echinococcosis and its relationship with IL-10, Parasite Immunology, 2011, 33, 692–696, J.-Y. Shan, W.-Z. JI, H.-T. LI, T. Tuxun, R.-Y. LIN1, & H. Wen) (**Table 3**).

Components	1×
H$_2$O	3 µl
Primer F	1 µl
Primer R	1 µl
SYBR Green Master	10 µl
Total	15 µl

Table 4. The PCR mix.

Activity	Number of cycles	Temp (°C)	Duration	Fluorescence acquisition
Preincubation	1	96	5 minutes	—
Amplification	45	95	10 seconds	—
		60	10 seconds	—
		72	20 seconds	Single
Melting analysis	1	95	5 seconds	
	1	55	1 minute	
	1	97	—	Continuous
Cooling	1	40	10 seconds	—

Table 5. Staged purification program.

Amplicons should be brought to a concentration of 10 µM and cDNA samples at a concentration of 25 ng/µl.

In a 0.5 mL Eppendorf tube, prepare the PCR mix according to **Table 4**.

From this mix, place 15 µl each well of the multiwell plate, and then add 5 µl cDNA to the appropriate wells. Seal the plate with heat-resistant foil, and centrifuge at 1500 g for 2 minutes. After centrifugation, the plate will be inserted into the LightCycler 480, and the amplification program presented and detailed in this chapter will be used.

After amplification we recommend that the data be analyzed with the LightCycler 480 Software, Basic/Advanced Relative Quantitation (**Table 5**).

4. Explanations and discussion

A simple representation of the concept of a research (or knowledge) system in science and engineering can be imagined as a "black box," considered only in terms of inputs, outputs, and function of the system or process in that system.

The current state of knowledge from the perspective of myocardial immunomodulation during and after (possibly) cardiotoxic treatment with anthracycline (especially doxorubicin) can

be empirically exemplified by this term "black box." I mean, we own a "black box"; we know what goes into this "box"; we know as well what goes out DAR; we do not know what's going on inside this "box."

Thus, we know of the presence of an immunomodulating inflammatory process in response to the anthracycline/doxorubicin molecular response and reaction; we know "cardiotoxic cardiovascular remodeling," but we do not know in detail the mechanisms by which these specific phenomena or specific immunological activators are responsible.

Dilated cardiomyopathy, a common cause of heart failure, is characterized by progressive cardiac remodeling and a decline in cardiac function. Currently, in the literature, the 5-year mortality rate for patients with dilatation cardiomyopathy is 50%. While many studies have documented that dilated cardiomyopathy has both idiopathic and genetic origins, it is being attempted at international academic medical level to demonstrate that inflammation of different origins and manifestations also induces dilated cardiomyopathy. Doxorubicin (Dox) is an effective antitumor agent. Despite its use as a common chemotherapeutic agent, the use of Dox may lead to cardiotoxicity.

Multiple intravenous treatments with doxorubicin over a period of several months have been shown to induce cardiomyopathy in mice and cardiomyopathy in humans [8]. Inborn immune response is the first line of defense and is responsible for the immediate recognition and counteraction of microbial invasion or any agents considered harmful to the body. This component of the immune system consists mainly of phagocytes-macrophages and neutrophils—which ingest and kill pathogens and then transmit information to the adaptive immune system by producing cytokines and chemokinesis by presenting to the lymphocytes the microbial antigen, which leads to the development of a specific response.

This specificity of the adaptive immune response, which is mediated by B and T lymphocytes, is accomplished by somatic mutations and the selection of receptors that most accurately recognize microbial antigens. In contrast, the innate immune response uses "pattern recognition receptors" (PRRs), which recognize highly conserved microbial structures, allowing the host to quickly identify a wide range of pathogens without the somatic mutation time. Generally, each receptor recognizes a series of microorganisms based on ligand specificities. Some receptors also have endogenous ligands and play essential roles in homeostasis (Brown G.D. 2006). Key receptors involved in the recognition of infectious agents and products released by injured or "dying" cells are Toll-like receptors (TLRs).

Activation of innate immune system receptors is followed by rapid changes in gene expression, including genes for cytokines, chemokines, degradative enzymes, and enzymes responsible for the production of small molecule inflammatory mediators. Thus, the released cytokines and chemokines can activate and recruit other cells at the site of the infection or at the site of "the presence of a non-self" (in the case of doxorubicin both by direct effect on receptors and by synthesis and degreasing) finally leading to the activation of the adaptive immune response. In mammals, activation of TLR signaling induces both innate and adaptive immune responses. By phagocytosis of microbial pathogens or non-self agents, TLR membranes (TLR-2, TLR-4) are recruited by phagosomes and are thus activated (mature as self-contained components) through cellular or microbial wall components. This phagosome maturation is also regulated by intracellular signals transmitted by the TLR and ensures the selection of microbial antigens

and their presentation by MHC II molecules. It is imperative that immature innate responses be fine-tuned. Delayed or insufficiently comprehensive responses lead to a failure to control infection or overexpressed inflammatory response in our case (doxorubicin cure). However, excessive or inadequate inflammation may be harmful and even fatal. Hyperinflammatory responses that characterize the body's response to treatment with doxorubicin or other anthracyclines provide a paradigmatic example, such as that excessive inflammation leads to inflammatory bowel disease and arthritis. Endogenous ligands of TLR2 and TLR4 include extracellular matrix products (hyaluronate and heparan sulfate) and molecules released by dead or injured cells (HMGB1, fibronectin, heat shock proteins, fibrinogen, and low density lipoproteins). Many of these molecules accumulate in patients' joints or other inflammatory sites, for example, in the myocardium with subsequent expression through an inflammatory process. The precise mechanism by which the TLR recognizes such a wide range of structures is not yet clarified (Marshak-Rothstein A, 2006). Multiple Toll-like receptors (TLRs) are expressed in cardiomyocytes, including TLR2 and TLR4. By these TLRs, cardiomyocytes respond to endogenous or exogenous signals that may influence pathophysiological responses to induce dilated cardiomyopathy.

Expression or activation of both TLR2 and TLR4 is often expressed in experimental and research models (both human and animal) by hypertension and with proven clinical heart failure [6]. Therefore, inhibition of TLR signaling may be of great therapeutic benefit for doxorubicin-induced cardiotoxic cardiomyopathy. Toll-like receptors (TLRs) recognize pathogens associated with molecular patterns such as lipopolysaccharides, peptidoglycan, bacterial lipoproteins, and oligonucleotides during inflammatory response. TLRs have interleukin-1 (IL1)-like response pathways as an intracellular signaling means leading to the nuclear localization of the kb/Rel-type nuclear transcription factor (NF).

Moreover, TLRs are expressed in various organs, such as the lung, brain, kidneys, heart, etc. Recent studies have suggested that the "activated myocardium" through mediated TLR signaling pathways in response to exogenous ligands induces myocardial dysfunction. Other studies analyzed by us for scientific documentation have also demonstrated that there are other signaling-mediated TLR pathways that are activated by endogenous signals such as heat shock protein and oxidative stress in cardiomyocytes isolated from ventricular level.

5. Conclusions

We must also keep in mind that it has recently been shown that TLR2 plays an important role in ventricular remodeling after myocardial infarction and also with cardiac specificity (TLR4 compared to it does not have a system/organ specificity). Doxorubicin (Dox) is an effective antitumor antibiotic in the anthracycline class. However, doxorubicin also induces cardiomyopathy leading to congestive heart failure, thus often limiting its clinical use.

Doxorubicin-induced cardiomyopathy is mainly caused by increased cardiac oxidant production. It has also been reported that treatment with doxorubicin causes the release of cytochrome C and results in the activation of caspase 3 and cellular apoptosis. These studies also indicate

that free radicals play an important role in doxorubicin-induced cardiotoxicity. Because oxidative stress after administration of doxorubicin has been identified to play a central role in cardiac dysfunction, we hypothesized that the expression (or overexpression exact) of TLR2 and TLR4 contributes to the pathogenesis of doxorubicin-induced cardiac dysfunction.

Conflict of interest

Nothing to declare.

Author details

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