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NAD(P)H:Quinone Oxidoreductase 1 Expression in Human Reproductive System and Mitomycin-C Cytotoxicity: A New Chapter for Old Compounds?

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Abstract

NAD(P)H:quinone oxidoreductase 1 (NQO1; DT-diaphorase; DTD) is a cytosolic two-electron reductase, and compounds of the family of quinones such as mitomycin C are efficiently bio-activated by this enzyme.

The observation that DT-diaphorase is over-expressed in many cancerous tissues compared to normal tissues has provided us with a selective target that can be exploited in the design of novel anticancer agents.

Because information about the cell-specific expression of DT-diaphorase was so scarce, this study was initiated to map the distribution of this enzyme in human tissues. We report here our findings concerning the reproductive organs.

Tissue samples taken from various components of the human reproductive system were analysed for expression of DT-diaphorase by immunohistochemistry.

We found a strong expression of this enzyme in testicular stromal cells (Leydig's cells) and in the epithelium of the epididymis and Fallopian tube.

These results suggest that quinones bio-activated by DT-diaphorase may be toxic to the reproductive system and cause clinical problems due to testosterone deficiency in men and infertility in both sexes. The implications of these observations need to be considered in pre-clinical evaluation of new anticancer quinones and in patients treated with these compounds.

Keywords: DT-diaphorase; NQO1; Cancer; Mitomycin toxicity; Quinones; immunohistochemistry; Infertility-male etiology; Infertility-female etiology; Reproductive system

Introduction

Alkylating agents have been used in cancer chemotherapy for over 50 years [1] and are still among the most widely administered and effective anti-tumour drugs. They are reactive chemicals that form covalent bonds with a variety of chemical groups on essential cellular molecules, but their reaction with DNA appears to be the primary event that causes cell death. These compounds are most toxic towards rapidly dividing cells, and side effects are therefore predicted to be against the bone marrow, gastro-intestinal mucosa, hair follicles and seminiferous tubules [2]. Quinones are a distinct class of alkylating agents that need to be activated by metabolic reduction [3]. The same side effects have been described for mitomycin C as for other alkylating agents except gonadal toxicity. In fact, no evidence has been presented for a possible toxicity of anticancer quinones on the reproductive system. Mitomycin C, the prototype of this group, requires enzymatic activation by reduction of the quinone group; this activation is NADPH-dependent and when activated, mitomycin produces cross-links in DNA. The specificity of agents of this type against tumour cells depends on the activity of the bio-reductive enzyme DT-diaphorase (NQO1; NAD(P)H:quinone oxidoreductase 1; DTD). DTD-rich cells have been shown to be more sensitive [4-6] to these agents. DT-diaphorase over-expression has been demonstrated in many cancerous tissues compared to normal tissues [7,8]. This allows the generation of higher concentrations of reactive compounds at the tumour site by the utilisation of DTD-activated pro-drugs. Compounds such as mitomycin C, streptonigrin, MeDZQ, EOg, tirapazamine and RH1 are efficiently bioactivated by this enzyme [4,5,9-15]. RH1 is the compound selected for clinical development by the Cancer Research Campaign (CRC) and the NCI, and after its preclinical evaluation [16], it successfully underwent phase 1 evaluation [17].

Physiologically, DT-diaphorase is believed to protect normal cells against redox cycling and oxidative stress by the reductive detoxification of quinones and their derivatives.

Quinones such as mitomycin C and streptonigrin have been used for 40 years and are active against head and neck, breast, gastro-intestinal and lung cancers. Despite the introduction of new families of antitumour drugs, mitomycin C is still widely used. It is suggested that the antitumour activity of such compounds could be enhanced by selective induction of DT-diaphorase in tumour cells compared to normal cells [18,19]; DTD can also be induced by dietary compounds [20]. A polymorphism of the gene encoding this enzyme has been described [21,22], and this variant is associated with reduced DT-diaphorase activity and resistance to anticancer agents requiring bio-reductive activation [23].

Despite the widespread interest in this enzyme because of its potential for selectively activating anticancer pro-drugs in tumour cells, which decreases the risk of toxicity to normal cells, very little information is available about its physiological distribution in the human body.

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In our pre-clinical evaluation of RH1, we performed a mapping of this enzyme in normal and tumoral human tissues, with the aim of identifying tumours over-expressing DT-diaphorase and therefore good targets for DTD bio-activated compounds, and also to predict potential toxicities [24,25]. We report our findings concerning the analysis of DTD expression in different components of the human reproductive system.

Materials and Methods

Human tissues

Archival samples of formalin-fixed paraffin-embedded tissues were supplied by the Institute of Pathology of Southern Switzerland, Locarno, Switzerland.

We analysed:

A. 10 bioptic samples of normal human testis (contra-lateral normal biopsies from patients undergoing hemi-castration because of testicular cancer).

B. 5 sections of normal human epididymis, that we found when analysing the testicular tumour sections of the above patients.

C. 10 bioptic samples of normal human ovary and Fallopian tube.

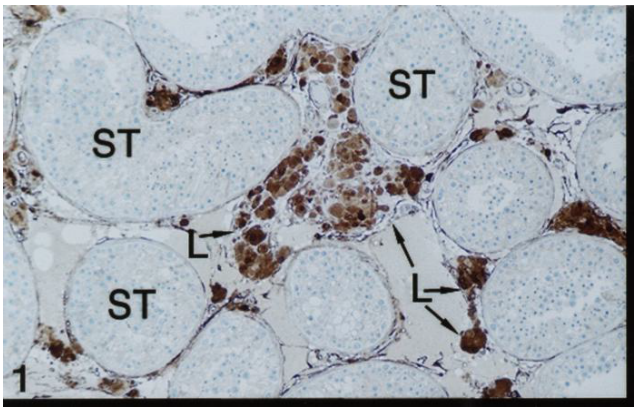

Figure 1: Immunohistochemical detection of DTD (brown staining) in human testis (low magnification). ST= seminiferous tubules, L= Leydig's cells.

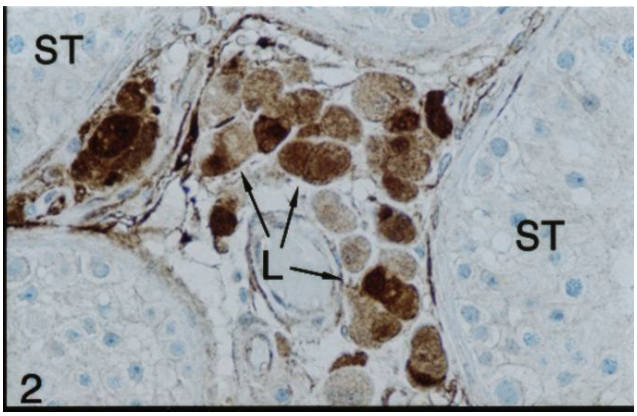

Figure 2: Immunohistochemical detection of DTD (brown staining) in human testis (high magnification). Score +4 limited to interstitial Leydig's cells (L), score 0 for seminiferous tubules (ST).

Methods

We performed an immunohistochemical analysis for the detection of DTD on tissue sections using anti-DTD antibodies.

Antibodies and Reagents: Anti-DTD monoclonal antibody (IgG1)-secreting hybridomas (clones A 180 and B 771) were derived from a BALB-c mouse immunized with purified recombinant human DTD protein. All hybridoma cell lines were grown in spinner flasks in RPMI medium containing 50units/ml penicillin, 50 µg/ml streptomycin, 1% L-glutamine and 10% fetal bovine serum in 5% CO_2 at 37°C to a concentration of 106 cells/ml. Hybridoma tissue culture supernatants were prepared by centrifugation at 1800 rpm for 10 min and then stored at -80°C.

Prior to use supernatants were centrifuged at 14,000 rpm for 5 minutes. We demonstrated specificity of our proprietary antibody by pre-absorbing it with antigen (human recombinant DTD), and showing that immunohistochemical staining was completely blocked. We also performed a Western analysis on tissue extracts to confirm immunohistochemical staining specificity.

Non-human reactive monoclonal mouse antibodies produced in tissue culture, subclass IgG1, were used as negative control reagent (Mouse IgG1 Negative Control Code No. X0931 DAKO A/S, Denmark).

Immunohistochemical method: Immunohistochemistry was performed on tissue sections (3µm) cut from archival paraffin blocks. Sections were de-paraffinized in xylene and rehydrated through graded alcohol to running water, then placed in citrate buffer pH 6.0 and microwaved for two 3-min cycles. Endogenous peroxidase activity was blocked by adding Peroxidase Blocking Agent (DAKO En Vision Kit, Carpinteria, CA 93013 USA). Non-specific binding was blocked by adding 20% normal rabbit serum. Serial sections of each tissue sample were then incubated with either anti-DTD or control antibodies for 30 minutes at room temperature. The secondary antibody was added for 30 minutes (DAKOKIT, labelled Polymer HRP anti-mouse: peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins). Immunodetection was performed using a substrate-chromogen solution (DAKOKIT, hydrogen peroxide and 3,3'-diaminobenzidine chromogen). Slides were counterstained with haematoxylin, dehydrated through graded alcohols, mounted and photographed.

Scoring of DTD immunostaining: The intensity of immunostaining of DTD (brown staining) was visually scored as 0 (negative), +1 (very weak), +2 (weak), +3 (strong), +4 (very intense) as previously reported for human lung cancers [26].

Results

All samples analysed were suitable for immunohistochemical analysis of DTD expression. There was no immunostaining in control sections when non-specific antibodies were used.

Immunohistochemical analysis of DTD in human normal testis.

Seminiferous tubules and Sertoli cells

There was no DTD expression (score 0) in any of the samples analysed (Figures 1 and 2). A normal spermatogenesis was present in 9 of 10 samples.

Testicular stroma

There was a very intense expression of DTD in the interstitial cells of the testis (Leydig's cells, score +4, Figures 1 and 2) of all samples

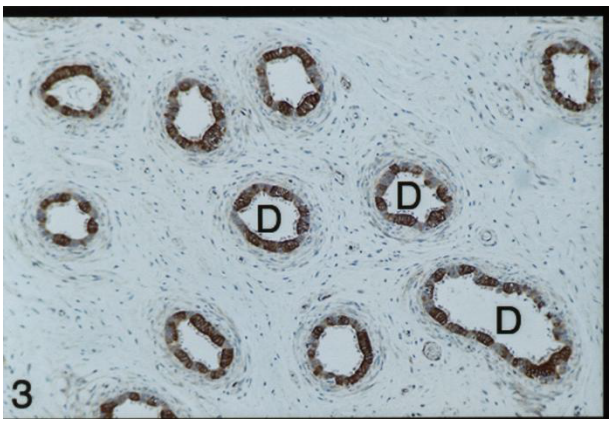

Figure 3: Immunohistochemical detection of DTD (brown staining) in human ductuli efferentes (low magnification). D= ductuli efferentes

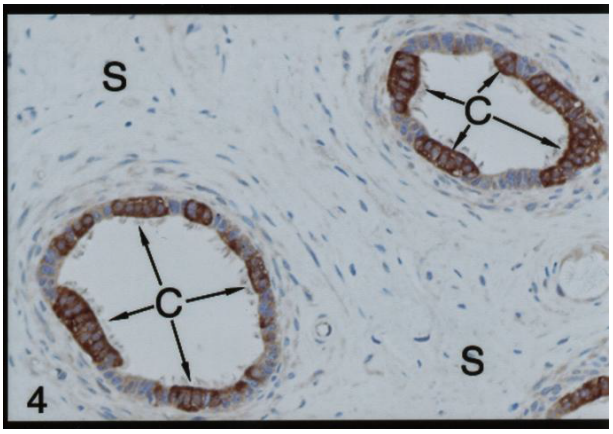

Figure 4: Immunohistochemical detection of DTD (brown staining) in human ductuli efferentes (high magnification). Score +4 limited to columnar ciliated epithelial cells (C, arrows), score 0 for stroma (S)

analysed. A strong to very intense (score +3/+4) immunostaining was also found in the endothelial lining of stromal vessels, but not in the connective tissue (score 0).

Immunohistochemical analysis of DTD in human normal ductuli efferentes and epididymis

Epithelial lining

There was a very intense (score +4) immunostaining in the columnar ciliated epithelial cells of ductuli efferentes (Figures 3 and 4), and a strong (score +3) immunostaining for DTD in the pseudostratified columnar epithelium of the epididymis in cells showing long microvilli (Figure 5).

Stroma

There was no or very weak staining for DTD in epididymal and ductuli efferentes stroma.

Immunohistochemical analysis of DTD in human normal ovary and Fallopian Tube

Normal ovary

We found a weak (score +2) immunostaining for DTD in the mesothelial lining on the surface of the ovary. The connective tissue stroma did not stain for DTD (score 0).

Fallopian tube

The Fallopian tube epithelium consists of a single layer of columnar cells of two types, ciliated and non-ciliated. The non-ciliated cells showed no staining for DTD (score 0), but the ciliated cells showed a very intense immunostaining (score +4, Figure 6). The Fallopian tube connective tissue stroma did not stain (score 0).

Discussion

Human male infertility secondary to treatment with cytotoxic chemotherapy has been widely described and is almost entirely a function of damage to the seminiferous tubule germinal epithelium [27], revealed by sperm count fall and follicle-stimulating hormone (FSH) increase, and histologically confirmed. Alkylating agents such as busulfan, chlorambucil, cyclophosphamide or procarbazine appear to be the ones most toxic to the germinal epithelium [28-31]. There is very little evidence of Leydig's cell insufficiency after cytotoxic chemotherapy

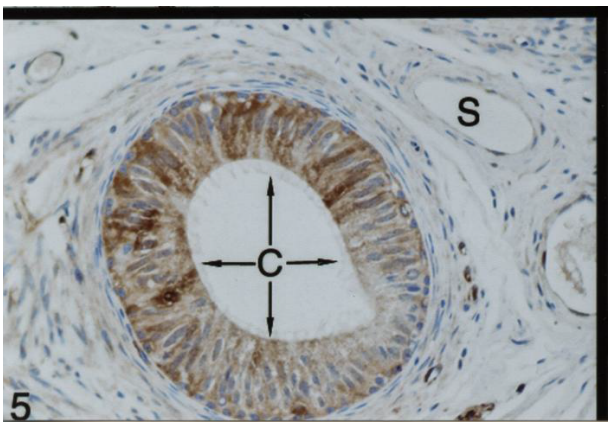

Figure 5: Immunohistochemical detection of DTD (brown staining) in human epididymis (E) (high magnification). Score +3 for the pseudostratified columnar epithelium of the epididymis (C, arrows), in cells showing long microvilli, and score 0 for stroma (S)

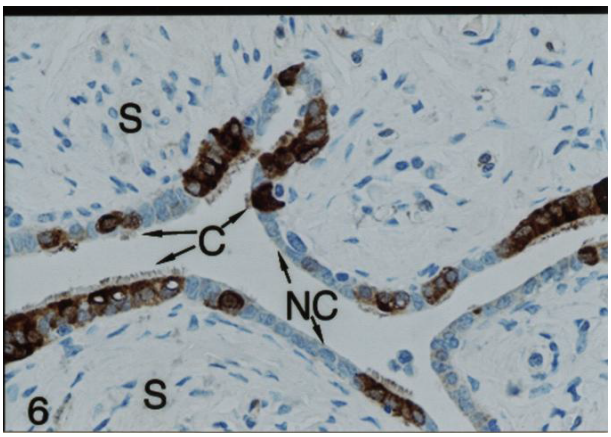

Figure 6: Immunohistochemical detection of DTD (brown staining) in human Fallopian tube (high magnification). Score +4 limited to ciliated epithelial cells (C, arrows), score 0 for non-ciliated epithelial cells (NC, arrows) and connective tissue stroma (S)

[32-35]. Previous studies propose "biological" evidence of Leydig's cell insufficiency as defined by an elevation of luteinizing hormone (LH) levels. No histological demonstration has as yet been reported. Moreover, these studies mainly concern patients treated with alkylating agents for Hodgkin's disease, and are retrospective. Pre-treatment values of gonadal function (testosterone, LH, FSH serum levels) are not available. It is known that Hodgkin's disease is associated with varying degrees of pre-treatment gonadal dysfunction and infertility [36-38]; it is hazardous to postulate Leydig's cell insufficiency in patients with Hodgkin's disease without knowledge of their pre-treatment gonadal function. A recent study showed no relevant evidence of Leydig's cell dysfunction [39]. Gonadal dysfunction after treatment for testicular germ cell cancer has also been widely investigated [40,41], but again this cancer is known to be associated before orchidectomy with spermatogenic and Leydig's cell dysfunction [42], and the specific role of chemotherapy is therefore difficult to assess.

Our study shows that DT-diaphorase is strongly expressed in various components of the human normal reproductive system, namely, in the interstitial Leydig's cells of the testis that secrete the male sex hormone testosterone and are responsible for the development of male secondary sexual characteristics and essential for the continued function of the seminiferous epithelium where spermatogenesis occurs, as well as in the epithelium of the epididymis, and that of the Fallopian tube that permits the migration of the ovum down the tube by a current of fluid secreted by the non-ciliated cells and propelled by the action of the ciliated cells. The physiological significance of DT-diaphorase in these cells probably has to be ascribed to a detoxifying protective action: from analysis of other human tissues we invariably also found an over-expression of this enzyme in two main categories of cells: hormone-producing cells and cells of body surfaces particularly exposed to noxious agents such as bowel mucosa and urinary tract epithelium. Quinone-induced toxicity to both the digestive and urinary systems have been widely described, in contrast to the scarcity of clinical evidence of such a toxicity on the human reproductive system. This could be explained by the fact that symptoms of testosterone deficiency such as fatigue, depression, loss of libido, impotence or anxiety, can be very aspecific [43]. Furthermore, Leydig's cells are among the most slowly dividing cells in the body, and because of this they may be relatively resistant to DNA-crosslinking agents. However, it is important that this potential toxicity be recognized, because this would render possible a more specific therapeutic approach to symptomatic patients, for instance, by testosterone replacement [44,45]. If further investigations confirm these observations, patients should benefit from pre-treatment counselling concerning their reproductive function, with respect to the chance for recovery of spermatogenesis, fertility, semen cryopreservation, and the possibility of their need for androgen replacement.

Surprisingly, although drugs such as mitomycin C have been in use for years no studies have focused on their effects on fertility and sexual function. A prospective evaluation of gonadal function in patients undergoing anti-cancer treatment with quinones, with serial screenings of testosterone, LH and FSH serum levels, is necessary to assess the magnitude of the problem. Patients who have been treated with these drugs and in whom symptoms potentially related to Leydig's cell dysfunction are seen should also be evaluated for sex hormone function, and this possible mechanism of toxicity should be taken into account in investigations of infertility in men or women after treatment with quinones.

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