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Dinitrosyl iron complexes with glutathione suppress experimental endometriosis in rats

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ABSTRACT

Dinitrosyl iron complexes (DNIC) with glutathione exert a cytotoxic effect on endometrioid tumours in rats with surgically induced experimental endometriosis. Intraperitoneal treatment of rats (Group 1) with DNIC (12.5 μmoles/kg, daily, for 12 days), beginning with day 4 after the surgical operation (implantation of two 2 mm-thick uterine fragments onto the abdominal wall) followed by 14-day keeping of animals on a standard feeding schedule (without medication) resulted in complete inhibition of the growth of endometrioid implants (EMI) in the majority of experimental animals. The ratio of mean EMI volumes in control and experimental rats of Group 1 was 14:1. In Group 2 rats, the use of a similar treatment protocol 4 weeks after surgery changed this ratio to 1.4:1. Noteworthy, the decrease of this ratio was irrelevant to deceleration of EMI growth at later periods after surgery. The histopathological analysis of EMI samples from experimental rats of Group 2 demonstrated complete disappearance of endometrial cysts suggesting a cytotoxic effect of DNIC on the tumours. The data obtained demonstrate that DNIC with glutathione and, probably, with other thiol-containing ligands hold considerable promise in the design of drugs for treating endometriosis in female patients.

1. Introduction

It has been established that paramagnetic mononuclear and diamagnetic binuclear dinitrosyl iron complexes (M- and B-DNIC) with thiol-containing ligands (cysteine or glutathione – RS^-) (formulas $\{(RS^-)_2Fe^+(NO^+)_2^+$ and $(RS^-)_2Fe_2^+(NO^+)_4^{2+}\}$, respectively) (Vanin et al., 2010; Borodulin et al., 2013) produce miscellaneous physiological and biochemical effects on animal and human organisms. Their biological action mimics those of nitrogen monoxide (NO), a universal endogenous regulator of metabolic processes, and its oxidised form, viz., nitrosonium ion (NO^+), and is based on the ability of DNIC to act as NO and NO^+ donors in living systems (Borodulin et al., 2013). By virtue of their ability to release NO, DNIC initiate vasodilation via a guanylate cyclase-dependent pathway (Mülsch et al., 1993; Vanin et al., 2007) and, as a consequence, provoke hypotension in animals and man (Lakomkin et al., 2007; Chazov et al., 2012). Moreover, DNIC inhibit platelet aggregation (Mordvintcev et al., 1986; Arkhipova et al., 2008), accelerate skin wound healing (Shekhter et al., 2007), induce penis erection (Andreev-Andriyevsky et al., 2011), and so on. By acting as NO^+ donors, DNIC with thiol-containing ligands initiate S-nitrosation of thiol-containing proteins responsible for programmed cell death and thus prevent apoptosis (Kim et al., 2000).

More recent studies demonstrated that in addition to their regulatory function, DNIC with thiol-containing ligands mimic cytotoxic effects of NO and NO^+ (Butler et al., 1995; Kleschyov et al., 2006). The latter are known to manifest cytotoxic activity during their augmented synthesis in body cells as a result of which their steady-state concentrations are maintained at a sufficiently high (>100 μM) level (Ignarro, 2000). Enhanced production of NO and NO^+ is provided by the inducible form of NO synthases and represents a specific response to activation of cell-mediated immunity and autoimmunity. The appearance of excess NO in various body cells and tissues is prerequisite to augmented synthesis of peroxynitrite, an extremely toxic compound formed during interaction of NO with the superoxide (Czabo et al., 2007). Steady-state concentrations of peroxynitrite and its decomposition products, e.g., hydroxyl radicals, nitrogen dioxide, etc., are high enough to overcome the endogenous protective system; therefore, excessive concentrations of NO have a cytotoxic effect on the organism. At low ($\sim$1 μM) steady-state concentrations of NO

Abbreviations: B-DNIC, binuclear dinitrosyl iron complex; EMI, endometrioid implant; EPR, electron paramagnetic resonance; GnRH, gonadotropin-releasing hormon; M-DNIC, mononuclear dinitrosyl iron complex; RNR, ribonucleotide reductase; RS-NO, S-nitrosothiol

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generated by constituitive (endothelial or neuronal) forms of NO synthases, the amount of peroxynitrite formed in the course of NO synthesis is too small to produce a cytotoxic effect.

A similar relationship between steady-state concentration and biological activity is characteristic of NO^+. In case of excessive steady-state concentrations of NO^+, the cytotoxic activity of NO^+ is controlled by the chaotic S-nitrosation of thiol-containing proteins (Kleschyov et al., 2006). As a result, the functional activity of the latter in the overall cascade of metabolic reactions appears to be strongly suppressed, resulting in apoptosis.

The regulatory effect of DNIC with thiol-containing ligands is determined by the chemical equilibrium between DNIC and their constituent components as shown in Scheme 1 for M-DNIC (Vanin et al., 2010; Borodulin et al., 2013):

$$(RS^-)_2\,Fe^+(NO^+)_2 \Longleftrightarrow Fe^{2+} + NO + \frac{RS^- + NO^+}{RS\text{-}NO} + RS^-$$

A similar pattern is characteristic of B-DNIC (Vanin et al., 2010; Borodulin et al., 2013). Interactions of NO or NO^+ with their biological targets, such as heme-containing enzymes (e.g., guanylate cyclase) or thiol-containing proteins possessing a high affinity for NO and NO^+, respectively, are terminated by a transfer of NO or NO^+ to their target proteins as a result of which their functional capabilities become activated or inhibited. In the absence of these proteins, DNIC with thiol-containing ligands retain NO and NO^+ by preventing their interactions with the superoxide (NO) or hydrolysis (NO^+) (Vanin et al., 2010; Borodulin et al., 2013).

A different situation takes place during interactions of DNIC with exogenous iron chelators, e.g., bathophenanthroline disulfonate or ethylenediamine tetraacetate, which result in the decomposition of DNIC and a release of significant amounts of NO and NO^+. For the first time, the cytotoxic effect of DNIC induced by these iron chelators was established in our studies on cultured HeLa cells preincubated with DNIC with cysteine or glutathione (Gilliano et al., 2011). Interestingly, DNIC themselves did not manifest any cytotoxic activity towards HeLa cells; however, the addition of iron chelators to the incubation medium caused their apoptotic death.

It is not improbable that similar effects may be produced not only by exogenous, but also by endogenous iron chelators. The latter are generated in rapidly proliferating cells and tissues supplying living organisms with iron. Interactions of iron chelators with DNIC localised in proliferating cells are prerequisite to the appearance of significant amounts of NO and NO^+, which may result in the apoptotic death of cells.

The main objective of this study was to examine cytotoxic effects of DNIC with glutathione on rats with experimental endometriosis using endometriotic implants (EMI) (tumour-like neoplasms formed on the surface of the abdominal wall due to uncontrolled growth of uterine epithelium) as a model of a rapidly proliferating tissue. Endometriosis is a rapidly progressing medical condition, which affects $>10\%$ of the female population of reproductive age (Mahmood and Templeton, 1991; Eskenazi and Warner, 1997; van Langendonckt et al., 2008). This disease is induced by implantation of endometrial cells with subsequent fast proliferation of endometrioid tissue (EMI) outside the uterine cavity, most commonly in the abdominal cavity. A search for drugs manifesting cytotoxic activity against EMI and based on a model of experimental endometriosis in animals is a currently central task.

Our previous summertime (June–July) experiments on rats with surgically induced endometriosis established that intraperitoneal injections of DNIC with glutathione (10 μM/kg) (daily, for 12 days, beginning with week 4 after the first surgical operation) decreased more than twofold the size of EMI together with complete disappearance of additive small-size endometrioid tumours (Adamyan et al., 2013). We hypothesised that the cytotoxic effect of DNIC with glutathione on EMI and additive endometrioid tumours is a result of decomposition of DNIC initiated by iron chelators released from rapidly proliferating EMI and additive small-size endometrioid tumours.

The main objective of this study was to compare the ultimate effects of DNIC with glutathione on EMI estimated one month after surgery, when the growth of EMI was complete, and to monitor this effect at the stage of their most intensive growth (12 days, beginning with day 4 after the first surgical operation).

2. Materials and methods

2.1. Materials

Reduced glutathione and cysteine were purchased from Sigma (St. Louis, USA). Ferrosulfate was from Fluka (Buchs, Switzerland). Gaseous NO was obtained by reaction of ferrosulfate with sodium nitrite in 0.1 M HCl with subsequent purification gaseous NO by low-temperature sublimation in an evacuated glass system.

2.2. Synthesis of DNIC with glutathione.

The synthesis of DNIC with glutathione was based on the ability of gaseous NO to bind to Fe^{2+} and glutathione in aqueous solutions at neutral pH (McDonald et al., 1965).

2.2.1. Protocol – synthesis of DNIC with glutathione

The synthesis was run in a Thunberg vessel (total volume 100 ml) at the Fe^{2+}: glutathione molar ratio 1:2 and under gaseous NO pressure 100 mm Hg. The solutions of $FeSO_4$ (0.5 ml) in distilled water (pH 5.5) and glutathione (4.5 ml) in 15 mM HEPES buffer (pH 7.4) were loaded into the upper and lower chambers of the Thunberg vessel, respectively; NO was loaded into the deaerated vessel. The Fe^{2+} + glutathione solutions were mixed in the presence of NO upon continuous shaking; NO was evacuated 5 min after mixing. This treatment stimulated the formation of B-DNIC, which represented a glutathione ether of Roussin's red salt (Vanin et al., 2010). The concentration of B-DNIC formed thereupon was determined from the concentration of $FeSO_4$ added to the solution and was found to be equal to 2.5 mM of B-DNIC or 5 mM (as calculated per one iron atom in B-DNIC).

The addition of a 20-fold glutathione excess to B-DNIC solutions and the increase of pH to 10–11 initiated the decomposition of binuclear DNIC and their further conversion into mononuclear paramagnetic DNIC with glutathione (Vanin et al., 2010). Their EPR signal was characterised by the following values of the g-factor: $g_\perp = 2.04$, $g_\parallel = 2.014$, $g_{aver.} = 2.03$ (the latter is commonly referred to as the 2.03 signal as regards its $g_{aver.}$ value) (Vanin et al., 2010). A similar conversion of binuclear DNIC with thiol-containing ligands was observed during interaction of the latter with thiol groups of proteins and yielded mononuclear paramagnetic protein-bound DNIC (Vanin et al., 1998).

2.3. Animals

Experimental endometriosis (EM) was induced in female Wistar rats (180–200 g) supplied by the «Stolbovaya» Nursery (Russia). All the rats tested in this study were in the proestrus phase. The experiments were carried out in full conformity with the Guidelines of the Geneva Convention "International Guiding Principles for Biomedical Research Involving Animals" (Geneva, 1990).