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EDITORIAL

TNFalpha in the Pathogenesis of Diabetes-Induced Embryopathies: Functions and Targets

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■ Abstract

Hyperglycemia-induced increase in the production of reactive oxygen species (ROS) is proposed to be an initial step in the pathogenesis of diabetes-induced spontaneous abortions and structural inborn anomalies. However, the subsequent steps in this process are incompletely understood. One of the key molecules involved is tumor necrosis factor-alpha (TNFα): its expression is regulated by ROS and it regulates ROS production in turn. This cytokine has been the focus of many studies addressing the mechanisms of different forms of diabetes-induced embryopathies, such as early pregnancy loss, inborn anomalies, fetal growth retardation as well as some pathologies appearing during adult life. In this review, we analyze the results of these studies and discuss how TNFα may regulate the response of pre- and post-implantation stage embryos to diabetes-induced detrimental stimuli. The data presented in this review suggest that TNFα may play a dual role in the pathogenesis of diabetes-induced embryopathies. It may act both as a mediator of diabetes-induced embryotoxic stimuli leading to the death of peri-implantation stage embryos and, possibly, as a suppressor of diabetes-induced apoptosis in post-implantation stage embryos. It also appears that TNFα fulfills these functions via interaction with leukemia inhibitory factor (LIF) and the transcription factor NF-κB. These molecules are presently considered as attractive targets for the treatment of diabetes-induced complications. Therefore, further studies addressing their role in the mechanisms underlying diabetes-induced embryopathies are needed to evaluate the safety of such therapies for diabetic women of childbearing age.

Keywords: diabetes · embryopathy · pregnancy loss · inborn anomalies · TNFalpha · NF-kappaB · LIF

Introduction

Spontaneous abortions and structural inborn anomalies are the main complications of diabetic pregnancy [1]. Early epidemiological observations revealed that the incidence of these adverse pregnancy outcomes correlates with high blood glucose and glycosylated hemoglobin (HbA1c) levels [2-4]. Rigorous glycemic control can reduce the rate of these complications in diabetic pregnancies [5], but the rate is still three to fivefold greater than for non-diabetic pregnancies [6].

In recent years, there has been considerable progress in our understanding of the etiology and pathogenesis of diabetes-induced embryopathies. Until recently, the role of glucose as a causative factor for diabetes-induced inborn anomalies remained questionable. About 30 years ago, Ornoy and Cohen were the first to suggest that glucose may have a teratogenic effect *in vivo* [7]. Later, the hypothesis was developed that glucose is not teratogenic *per se* but rather a marker for other diabetes-generated teratogens such as ketone bodies, triglycerides, branched chain amino acids, sorbitol or glycated proteins [8, 9]. In our *in vivo* study, we

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demonstrated that the occurrence of severely malformed fetuses in litters of streptozotocin (STZ)-induced diabetic ICR mice is associated with glucose levels >27.8 mmol/l [10]. This result appeared to support the latter hypothesis. On the other hand, this study did not detract from the suggestion made by Ornoy and Cohen that episodes of elevated glucose levels can induce congenital anomalies. The reason is that malformed fetuses were observed also in litters of STZ-treated mice, which were considered to be non-diabetic at the end of pregnancy [7]. Recently, a study performed by Loeken's team provided convincing evidence that elevated glucose levels are the main etiological factor for diabetes-induced inborn anomalies [11].

Most researchers now accept that hyperglycemia-induced increase in the production of reactive oxygen species (ROS) is an initial key event in the pathogenesis of diabetes-induced structural anomalies [12-15]. However, as ROS are capable of regulating numerous intracellular signal transduction pathways [16], subsequent pathological events seem to be far from completely understood. One of the key molecules involved is tumor necrosis factor-alpha (TNFα): its expression is regulated by ROS and it regulates ROS production in turn [17]. This cytokine has been the focus of many studies addressing the mechanisms underlying diabetes-induced embryopathies [18, 19]. In this review, we analyze the results of these studies and discuss how and via which targets TNFα may regulate the response of pre- and post-implantation stage embryos to diabetes-induced detrimental stimuli.

TNFα and diabetes-induced early pregnancy loss

TNFa mediates diabetes-induced death of early embryos

Experiments in STZ- or alloxan (ALX)-induced diabetic female mice [10, 20, 21] or rats [22] showed that their pregnancy rate (the proportion of mated females that become pregnant) is significantly lower than that of their non-diabetic counterparts. In these studies, neither implantation sites nor resorptions were found in the uteri of mated diabetic but non-pregnant females. These results suggested that pregnancy failure in such females resulted from diabetes-induced early embryonic death, i.e. death of the pre- or peri-implantation stage embryos. To clarify this question, we evaluated the pregnancy rate in STZ-induced diabetic mice on days 4 (the end of the pre-implantation period) and 8 (the end of the implantation period) of pregnancy [23]. We found that the pregnancy rate was identical in diabetic and non-diabetic females tested on day 4, but not on day 8, of pregnancy. On day 8 of pregnancy, diabetic females exhibited a decrease in the pregnancy rate. We concluded that pregnancy failure in those mice resulted from death of peri-implantation stage embryos.

Our study also revealed that the pregnancy rate in STZ-induced diabetic TNFα knockout mice was lower than that in non-diabetic females but far higher than that recorded in their TNFα-positive counterparts [23]. The critical outcome of this observation was that the cytokine may be a central component in the mechanisms underlying diabetes-induced death of early embryos.

Does TNFa mediate the diabetes-induced death of peri-implantation embryos by affecting the embryo or uterus?

Based on the fact that diabetes-induced early pregnancy loss results from death of peri-implantation embryos, two scenarios mediated by TNFα are conceivable. Either the cytokine affects the ability of pre-implantation stage embryos to implant in the uterus or it impairs the ability of the uterus to establish implantation.

The first scenario is supported by data demonstrating the ability of TNFα to affect early embryos adversely. Indeed, it has been observed that rat blastocysts exposed to TNFα exhibited decreased cell proliferation [24] and an increased rate of blastomere apoptosis [25]. The same impairments have been observed in cultured mice and cattle blastocysts exposed to TNFα [26, 27]. The relevance of these findings to maternal diabetes was shown in a study by Pamper and coworkers [28]. In this study, the authors incubated rat blastocysts in a diabetic culture medium and found an improved growth when pretreating the blastocysts with anti-sense oligonucleotides that blocked TNFα receptors. It is also worth noting that suppression of cell proliferation and excessive apoptosis were observed in pre-implantation mouse and rat embryos developing in a diabetic environment (references in [29]).

Although the above mentioned data show that TNFα can injure early embryos, a scenario, in which these injures are held responsible for early pregnancy loss in diabetic females, seems questionable. There is a considerable inter- and intra-litter variability in the susceptibility of embryos to detrimental stimuli [30]. If TNFα-induced injures in embryos were responsible for early pregnancy loss, then we would observe a significant decrease in the implantation rate in pregnant diabetic females at any time point after completing the implantation period. Although three studies found

lower implantation rates [22, 31, 32], in most studies the rate did not differ from that observed in non-diabetic females [7, 10, 20, 21, 23, 33-43]. When blastocysts were exposed to diabetic environments or TNFα, the cellular deficit in these blastocysts was mostly at the expense of the inner cell mass (ICM) - the cells that form the fetus - but not of the trophectoderm (TE) cells that ensure implantation of the blastocyst into the uterine wall [44]. Consistent with this finding, embryo transfer experiments revealed that TNFα-treated mouse blastocysts implant practically at the same rate as control blastocysts but exhibit a higher incidence of resorptions (i.e. post-implantation death) [29]. The same result was obtained in an embryo transfer study in non-obese diabetic (NOD) mice. While the implantation rate of NOD blastocysts transferred to the uteri of ICR mice (control) did not differ from that of ICR embryos transferred to ICR uteri, the level of resorptions of the former was significantly higher than that of the latter [45]. In the light of these findings, it seems reasonable to suggest that diabetes-induced alterations in pre-implantation embryos mainly disturb their ability to develop rather than implant into the uterus.

The second possible scenario explaining early pregnancy loss proposes that the death of peri-implantation embryos in diabetic females results from TNFα-induced injuries in the peri-implantation uterus. This view is based on data demonstrating that TNFα mRNA and protein are overexpressed in the uterine cells of diabetic females from the initiation of implantation and onwards ([42] and references in [18, 19]). As TNFα is able to activate the death receptor-mediated apoptotic signaling cascade [46], these observations suggest that the apoptotic process in the uterus, which is crucial for the implantation of the embryo [47], may be distorted in diabetic mice. The suggestion is supported by studies showing that mouse uterine epithelial WEG-1 and human endometrial HEC-1 cells exposed to TNFα or cultured in a diabetic condition exhibit a decreased viability and several apoptotic markers [18]. A mechanistic role for the uterus in diabetes-induced early pregnancy loss is also suggested by data demonstrating a lower implantation rate of ICR blastocysts (control) transferred to NOD uteri compared with that of ICR blastocysts transferred to ICR uteri [45]. Finally, this scenario may explain why the death of peri-implantation embryos in some diabetic females is not accompanied by a decrease in the implantation rate in diabetic females who retain pregnancy.

In summary, the observations mentioned above appear to suggest that the peri-implantation uterus rather than the pre- or peri-implantation embryo itself is the target and that TNFα may play a role in mediating diabetes-induced early pregnancy loss. This view is further discussed in the following section.

Is the TNFa-induced apoptosis in the uterus a mechanism of diabetes-induced early pregnancy loss?

For successful implantation, the temporal pattern and intensity of apoptosis in the peri-implantation uterus have to be tightly regulated [47]. Therefore, it is conceivable that TNFα-induced death receptor-mediated activation of apoptosis in the peri-implantation uterus may be harmful to implantation. However, it is important to bear in mind that TNFα-activated death receptor-mediated signaling cascade also activates the transcription factor NF-κB [48], which is a powerful negative regulator of apoptosis. Considerable evidence suggests that its activation protects cells against TNFα-induced apoptosis [49, 50]. A recent study in a mouse model of autoimmune type 1 diabetes demonstrated the anti-apoptotic role of NF-κB in pancreatic β-cells exposed to TNFα [51].

As to the role of NF-κB localized in the uterus, lack of activity on its part does not seem to impair implantation, as was observed in experimental mice where NF-κB activation has been blocked (references in [52]). At the same time, NF-κB activity in uterine cells is tightly regulated during implantation [53]. Furthermore, NF-κB was found to be activated in uterine cells exposed to TNFα and it was suggested that this event might be a transient NF-κB-dependent anti-apoptotic reaction [18].

In summary, excessive apoptosis in the peri-implantation uterus cannot be excluded as a possible cause for diabetes-induced early embryonic death. Nor can we exclude the possibility that TNFα-activated death receptor-mediated signaling in uteri of diabetic females retaining pregnancy may be an event supporting implantation via the activation of NF-κB-mediated anti-apoptotic signaling. In the latter case, it is possible that TNFα mediates diabetes-induced early pregnancy loss by affecting mechanisms that regulate uterine receptivity for blastocyst implantation. We discuss these mechanisms in the next section.

LIF as a possible TNFa target

One of the main mechanisms ensuring uterine receptivity is associated with the function of leukemia inhibitory factor (LIF) [54]. Earlier, we hypothesized that LIF may be involved in pathways underlying stress-induced TNFα-mediated early pregnancy loss

[55]. In this section, we discuss this hypothesis in more detail.

The level of LIF in the uterus is tightly regulated, reaching a peak at the time of implantation [54]. In mice, implantation does not occur in LIF$^{-/-}$ uteri, whereas LIF null blastocysts develop successfully to term in wild-type females [56]. LIF is able to trigger several signaling pathways, including the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway, which is currently suggested to be essential for the acquisition of uterine receptivity [54, 57]. LIF triggers this pathway by binding to its receptors (LIFR), followed by activation of the JAK family kinases, which, in turn, activate STATs transcription factors, in particular STAT-3 [57]. The importance of STAT-3 for the acquisition of uterine receptivity has been demonstrated [58] and it is now known that the JAK/STAT signaling pathway must be activated for successful implantation [54].

TNFα is able to induce LIF expression in many cell types, including human endometrial epithelial and stromal cells in a concentration and time-dependent manner (references in [55]). TNFα is also a powerful activator of NF-κB, which, in turn, activates TNFα expression [49, 50]. It has been demonstrated that the promoter region of the LIF gene contains an NF-κB binding site [59] and that NF-κB may mediate TNFα-stimulated LIF production in human endometrial epithelial cells [60]. NF-κB was also suggested to be involved in the regulation of STAT-3 DNA-binding [57]. Based on these data, we may propose a model, in which TNFα overexpression alters the function of LIF in the uterus of diabetic mice leading to the death of peri-implantation embryos (Figure 1).

Yet, there are still many unanswered questions. One question is whether diabetes-induced TNFα-mediated pregnancy loss may result from an increase or decrease in LIF production in the uterus. Indeed, some increase in TNFα and decrease in LIF expression were observed in fluid derived from uterine irrigation of women with recurrent failed embryo transfer [61]. At the same time, data exist suggesting that LIF overexpression in the uterine lumen may be also harmful to implantation [62]. The condition is complicated by the complex organization of the LIF gene that is involved in translation of intracellular and extracellular proteins with distinct cellular activities [63]. Furthermore, it is possible that the direction of the LIF secretion by polarized uterine epithelium may be a factor determining the outcome of implantation. It has been suggested that secretion towards the basal cells is necessary for implantation, whereas secretion in the apical direction towards the uterine lumen may be harmful for implantation [62].

In addition, it is unclear, whether LIF-mediated regulation of the implantation rate of mouse and rat embryos is an "all-or-nothing" phenomenon. The answer to this question is important because it will make it possible to estimate the extent to which other factors regulating uterine receptivity may be involved in mechanisms underlying diabetes-induced early pregnancy loss.

Finally, TNFα does not seem to be the only molecule capable of affecting the function of LIF in the diabetic uterus. In an experimental study, the gene encoding LIF was found to be a potential target for the tumor suppressor gene p53 [64]. The authors observed that the dramatic decrease in pregnancy rate in p53$^{-/-}$ female mice, as compared to that recorded in p53$^{+/+}$ females, was accompanied by a decrease in uterine LIF mRNA expression. Although there is no proof for changes in p53 expression in uterine cells of diabetic mice, studies demonstrating that p53 is activated in the process of hyperglycemia-induced apoptosis in pre-

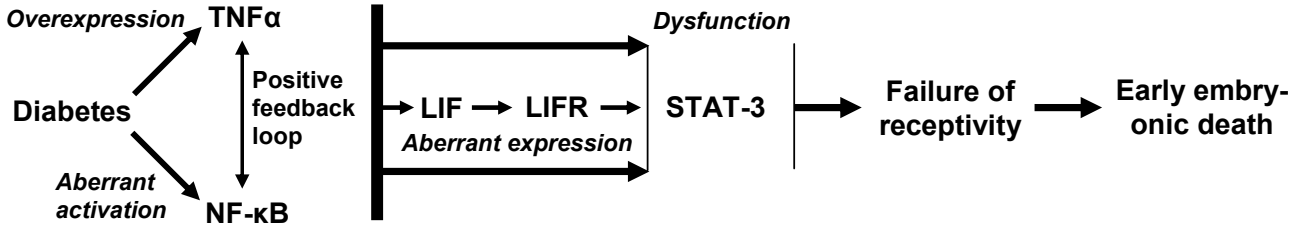

Figure 1. A model of a pathway triggered by TNFα in the uterus of diabetic mice, which can lead to the death of peri-implantation embryos. Diabetes increases TNFα expression and activates NF-κB in the uterus. These effects may be boosted via a positive feedback loop between these molecules and followed by TNFα- and/or NF-κB-induced dysregulation of molecules, such as LIF, LIFR and STAT-3, which have a critical function for uterine receptivity.

implantation stage embryos [65] suggest that such changes are possible. In the light of evidence that not only decreased but also increased production of LIF in the uterus may be harmful for implantation [62], it is important to investigate the role of p53 as a possible regulator of LIF secretion in the peri-implantation uterus of diabetic females.

TNFα and diabetes-induced inborn structural anomalies

Targets for diabetes-induced teratogenic injuries

The possibility that the hyperglycemia-induced injury of pre-implantation embryos may predispose them to malformations later in embryogenesis [66] or even directly result in structural malformations [67] can not be excluded. Studies using whole embryo culture suggest that diabetes-induced neural tube defects (NTDs) (exencephaly, anencephaly, microcephaly, spina bifida) result from a hyperglycemia-induced injury of gastrulation and neurulation-stage embryos [68, 69]. It has been proposed that alterations in the histiotrophic function of the visceral yolk sac (VYS) may also be involved in the pathogenesis of hyperglycemia-induced malformations in these embryos [70]. However, subsequent studies [69] have failed to confirm a relationship between altered VYS hystotrophic function and the occurrence of structural anomalies. Presently available data suggest that the embryo at the neural tube closure stage (between 8.5 and 10.5 embryonic days in mice) is the main target of the diabetes-induced teratogenic stimuli.

A possible role for TNFa in diabetes teratogenesis

Evidence for the role of TNFα diabetes-induced malformations in embryos was generated in experimental studies with diabetic TNFα knockout mice. In these studies, the proportion of malformed fetuses in diabetic $TNF\alpha^{+/+}$ mice was lower than in diabetic $TNF\alpha^{-/-}$ mice [23]. On the assumption that TNFα acts as a mediator of diabetes-induced death of peri-implantation stage embryos, the most acceptable explanation for this phenomenon is that death decreases the proportion of teratologically sensitive $TNF\alpha^{+/+}$ embryos in a population exposed to a diabetes-induced teratogenic effect taking place after implantation. However, the number of implantation sites in diabetic $TNF\alpha^{+/+}$ mice was practically identical to that in non-diabetic $TNF\alpha^{+/+}$ mice.

Another explanation for TNFα-induced malformations might be associated with an increased death of post-implantation $TNF\alpha^{+/+}$ embryos with severe structural anomalies. However, we observed that the number of living fetuses in diabetic $TNF\alpha^{+/+}$ mice did not differ significantly from that in $TNF\alpha^{-/-}$ mice. Together, these results implied that TNFα-mediated mechanisms aimed at preventing the formation of diabetes-induced structural anomalies may operate in post-implantation embryos.

Possible mechanisms underlying the TNFa-regulated response to diabetes-induced teratogenic injury

It has been proposed that diabetes-induced NTDs arise from incomplete closure of the neural tube. For the neural tube to be formed, the apoptotic process involved in its formation has to be tightly regulated [71]. A large number of studies demonstrate excessive apoptosis in the brain of early post-implantation stage embryos that develop in a diabetic environment and exhibit open neural folds [38, 72-75]. Interestingly, excessive apoptosis was also observed in the VYS of these embryos [76]. While the role of excessive apoptosis in the VYS in the pathogenesis of diabetes-induced NTDs remains unclear, excessive apoptosis in the embryonic brain appears to be mechanistically linked to this pathology. Indeed, a study performed by Loeken's team [38] revealed that excessive apoptosis in the embryos of diabetic mice exhibiting NTD is accompanied by reduced expression of the Pax3 gene regulating neural tube closure in the area of the mid- and hindbrain. Subsequent experiments in embryos obtained from crosses of $Pax3^{+/-}/p53^{+/-}$ males and females demonstrated that the loss of p53 function, by genetic or chemical means, prevented both apoptosis and NTDs caused by Pax-3 deficiency [77]. Based on these data, a model was proposed, in which excessive glucose metabolism inhibits the expression of Pax3 [6]. This, in turn, leads to the activation of p53-dependent apoptosis of the neuroepithelium and, consequently, to the formation of NTDs.

Our study in diabetic TNFα knockout mice revealed that the level of excessive apoptosis in the brain of $TNF\alpha^{-/-}$ embryos was higher than in the brain of their TNFα-positive counterparts [23]. Recalling that TNFα may counteract diabetes-induced apoptosis, we need to ascertain the mechanism by which this preventive function of TNFα is carried out. We hypothesized that NF-κB is a target via which TNFα may positively regulate the resistance of post-implantation embryos to diabetes-induced apoptotic stimuli [23].

As mentioned above, in most cell types NF-κB exists in an inactive form in the cytoplasm but is transcriptionally active in post-implantation stage embryos.

The first evidence to demonstrate the functional role of NF-κB in normal embryogenesis was obtained from studies in mice lacking the p65 subunit of NF-κB [78]. The embryos died on days 14-15 of pregnancy and this detrimental event was associated with massive hepatocyte apoptosis. Other experiments were carried out with mice lacking the inhibitory NF-κB (IκB) protein kinases 1 and 2 (IKK1 and IKK2), which are crucial for NF-κB activation [79]. The loss of NF-κB activity in these mice was associated with an increased incidence of embryos with exencephaly and excessive apoptosis in the neuronal epithelium.

The abovementioned studies suggested that NF-κB in organogenesis stage embryos acts as an apoptosis inhibitor. Teratological studies supported this suggestion. Indeed, experiments with thalidomide [80], cyclophosphamide (CP) [81] and valproic acid [82] imply that the suppression of NF-κB activity may be a mechanism by which the teratogens activate apoptosis in targeted embryonic structures. On the other hand, exposure of embryos to phenytoin resulting in nonclosure of the anterior neuropore was found to be associated with NF-κB activation [83]. However, not only activation but also suppression of apoptosis may adversely affect the process of neural tube formation [84]. Therefore, the study with phenytoin does not invalidate the finding that NF-κB works as an apoptosis inhibitor in embryos.

Our study in diabetic mice revealed that malformed TNFα$^{-/-}$ embryos exhibit a lower level of active NF-κB complexes than TNFα$^{+/+}$ embryos [23]. Because TNFα can be regarded as a powerful activator of NF-κB, we prpopose the following hypothesis: if NF-κB functions as a negative regulator of apoptosis during neural tube closure, then TNFα may act as a suppressor of diabetes-induced apoptosis by counteracting diabetes-induced suppression of NF-κB activity. Further studies are needed to investigate whether this hypothesis is correct. Nevertheless, it is worth noting that our data implicate a regulatory role of NF-κB on diabetes-induced apoptotic stimuli. These data were confirmed by a recent study, in which the increased incidence of malformations was associated with a decreased NF-κB activity in embryos of STZ-induced diabetic rats [85].

Teratological studies with cyclophosphamide (CP) are also interesting in this regard. The teratogenic potential of CP is mainly associated with DNA damage induced by its metabolites such as phosphoramide mustard and acrolein [86, 87]. CP is also capable of inducing ROS and oxidative stress [88], suggesting that the teratogenic mechanism of CP may be similar to that of diabetes. Our studies implying that TNFα-mediated activation of NF-κB in embryos is a mechanism increasing their resistance to CP [89, 90] support the hypothesis for a regulatory role of TNF-α-activated NF-κB in diabetes.

The model by Loeken to explain the mechanisms of diabetes-induced NTDs suggests that accumulation of p53 is a central event in the pathway underlying diabetes-induced excessive apoptosis [6]. Our recent study revealed that CP-induced excessive apoptosis is also mediated by p53 and, importantly, that p53 mediates CP-induced suppression of NF-κB DNA binding [91]. These data suggest that Loeken's model can legitimately be used to explain both diabetes-induced suppression of NF-κB activity and the function of TNFα as a protector against diabetes-induced teratogenic stress.

Conclusion

The data presented in this review suggest that TNFα may play a dual role in the pathogenesis of diabetes-induced embryopathies. It may act as a mediator of diabetes-induced embryotoxic stimuli leading to the death of peri-implantation stage embryos and as a suppressor of diabetes-induced apoptosis in post-implantation stage embryos. In addition, they suggest that molecules such as LIF and NF-κB may be critical players in the mechanisms determining the outcome of diabetes-induced embryopathic stress.

TNFα, LIF, NF-κB and molecules involved in NF-κB activation pathways are presently considered to be possible targets for the treatment of diabetes and diabetes-induced complications [92-94]. However, it is pointed out that therapy based on these molecules may have several detrimental consequences [95]. The problem may be aggravated, if the therapy results in modulation of a target molecule in the uterus and embryo. An increased incidence of spontaneous abortions and/or malformed offspring may be one of the unexpected side effects. Therefore, we need to learn more about the role of these molecules in the pathogenesis of diabetes-induced embryopathies.

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