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Oxidant regulation of gene expression and neural tube development: Insights gained from diabetic pregnancy on molecular causes of neural tube defects

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Abstract

Aims/hypothesis. Maternal diabetes increases oxidative stress in embryos. Maternal diabetes also inhibits expression of embryonic genes, most notably, *Pax-3*, which is required for neural tube closure. Here we tested the hypothesis that oxidative stress inhibits expression of *Pax-3*, thereby providing a molecular basis for neural tube defects induced by diabetic pregnancy.
Methods. Maternal diabetes-induced oxidative stress was blocked with α-tocopherol (vitamin E), and oxidative stress was induced with the complex III electron transport inhibitor, antimycin A, using pregnant diabetic or non-diabetic mice, primary cultures of neurulating mouse embryo tissues, or differentiating P19 embryonal carcinoma cells. *Pax-3* expression was assayed by quantitative RT-PCR, and neural tube defects were scored by visual inspection. Oxidation-induced DNA fragmentation in P19 cells was assayed by electrophoretic analysis.
Results. Maternal diabetes inhibited *Pax-3* expression and increased neural tube defects, and α-tocopherol blocked these effects. In addition, induction of oxidative stress with antimycin A inhibited Pax-3 expression and increased neural tube defects. In cultured embryo tissues, high glucose-inhibited *Pax-3* expression, and this effect was blocked by α-tocopherol and GSH-ethyl ester, and *Pax-3* expression was inhibited by culture with antimycin A. In differentiating P19 cells, antimycin A inhibited *Pax-3* induction but did not induce DNA strand breaks.
Conclusion/interpretation. Oxidative stress inhibits expression of *Pax-3*, a gene that is essential for neural tube closure. Impaired expression of essential developmental control genes could be the central mechanism by which neural tube defects occur during diabetic pregnancy, as well as other sources of oxidative stress. [Diabetologia (2003) 46:538–545]

Keywords Embryogenesis, diabetic pregnancy, oxidative stress, free radicals, neural tube defects, Pax-3, cell death, gene expression.

The offspring of women with diabetes mellitus are at increased risk for congenital defects [1, 2, 3, 4, 5, 6, 7, 8]. The incidence and severity of the defects are related to glycaemia within the first weeks of pregnancy, during which organogenesis is initiated [9, 10, 11, 12]. Reactive oxygen species (ROS) are increased in rat embryos exposed to excess glucose [13, 14, 15], which could be due both to increased oxidative metabolism and superoxide generation [15], the relative immaturity of the free radical scavenging system, [16] as well as inhibition of free radical scavenging pathways [13]. Since supplemental antioxidants [vitamin E, vitamin C, butylated hydroxytoluene (BHT), the glutathione (GSH) precursor, N-acetylcysteine], or transgenic overexpression of copper/zinc superoxide dis-

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Abbreviations: NTD, neural tube defect(s); MDA, malondialdehyde; ROS, reactive oxygen species; RA, retinoic acid; AA, antimycin A; GSH, glutathione; BHT, butylated hydroxytoluene.

mutase, can prevent structural defects caused by high glucose in rodent embryos [13, 14, 17, 18, 19, 20, 21, 22, 23], this indicates that hyperglycaemia-induced oxidative stress plays a causal role in diabetes-induced congenital defects.

Previously, we have shown that in mouse embryos, expression of *Pax-3*, a gene required for neural tube closure, is reduced, and neural tube defects (NTD) are increased, by maternal diabetes [24]. The excess glucose resulting from maternal diabetes is necessary and sufficient for this effect [25]. While there are likely to be multiple genes whose expression could be affected by maternal diabetes, the similarities between the structural defects seen in homozygous Splotch (*Sp/Sp*) embryos, which carry loss of function *Pax-3* alleles, and defects caused by diabetic pregnancy in humans and animal models (for example, open NTD, affecting closure of the cranial or caudal neuropore, and cardiac outflow tract defects, resulting from defective cardiac neural crest migration [1, 5, 26, 27, 28, 29]), suggests that Pax-3 deficiency might explain many of the neural tube and neural crest defects associated with diabetic pregnancy. Neural tube and neural crest defects occur with 100% penetrance in *Sp/Sp* embryos [26], indicating that there are no redundant pathways to compensate for the absence of Pax-3. Thus, in embryos in which maternal diabetes suppresses *Pax-3* expression to insufficient levels, NTD that phenocopy the *Splotch* mutation would be observed.

It is not known how excess glucose inhibits expression of *Pax-3*. Since excess glucose increases ROS in embryos [13, 14, 15], and oxidants can regulate cellular signalling pathways, including those that control transcriptional responses [30, 31], excess oxidants could interfere with signals that induce the expression of *Pax-3*. Here we tested this hypothesis by alleviating oxidative stress with antioxidant administration, and by inducing oxidative stress with an electron transport complex III inhibitor.

Materials and Methods

Mice. Diabetes was induced in 6- to 8-week-old female ICR mice (Taconic, Germantown, N.Y., USA) with the drug, streptozotocin (Sigma, St. Louis, Mo., USA), treated with insulin pellets (Linshin, Scarborough, Ontario, Canada), and monitored using a Glucometer Elite (Bayer, Mishawaka, Ind., USA) [24]. Insulin pellets allowed the streptozotocin-induced diabetic mice to maintain euglycaemia prior to pregnancy, but to become hyperglycaemic beginning on day 4.5 of gestation. Mice were mated, along with age-matched controls, to non-diabetic ICR males. Beginning on the day that a copulation plug was found (day 0.5), mice were fed either control chow (Lab Diet 5020, containing 0.006% α-tocopherol, Purina, St. Louis, Mo., USA) or chow supplemented with the water-soluble (+)-α-tocopherol succinate form of vitamin E (0.125%, w/w obtained from Sigma). This dosage of α-tocopherol succinate was found to be the highest non-toxic dosage in pregnant ICR mice (data not shown). Chow was provided ad libitum. There were no differences in consumption of control compared to supplemented chow (data not shown). To induce oxidative stress with antimycin A, a complex III electron transport inhibitor which increases mitochondrial production of superoxide and hydrogen peroxide [36, 37] mice were injected s.c. once on gestational day 7.5 with 3 mg/kg body weight antimycin A (Sigma Chemicals) dissolved in 25% (v/v) propylene glycol. Control mice were injected with 25% (v/v) propylene glycol/saline, or 25% (w/v) glucose dissolved in saline at approximately hourly intervals to maintain blood glucose greater than or equal to 17 mmol/l over a 10-h period [25]. Mice were killed on day 8.5 or 10.5. Embryos recovered on day 8.5 from each pregnancy were pooled and saved for assay of Pax-3 mRNA by relative quantitative RT-PCR, and decidua from each pregnancy were pooled for malondialdehyde (MDA) analysis. Embryos recovered on day 10.5 were scored for NTD. All procedures using animals conformed to the principles of laboratory animal care set forth by the NIH and were approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center.

Embryo Culture. Primary culture of embryos obtained on day 9.5 of gestation was carried out as described [25]. Briefly, minced tissues corresponding to two embryos (dissected free of extraembryonic membranes and manually disrupted to produce small tissue clumps) were cultured in triplicate in multiwell plates (Nunc, Roskilde, Denmark) coated with 1% gelatin. Tissues were cultured in DMEM (GIBCO, Rockville, Md., USA) plus 10% foetal calf serum (Sigma Chemicals) containing 7.5 mmol/l glucose (low glucose-containing media), or 25 mmol/l glucose (high glucose containing media), with or without DL-α-tocopherol acetate (Fluka, Buchs, Switzerland), or glutathione ethyl ester (Sigma Chemicals). α-tocopherol acetate was dissolved in 40% propylene glycol, diluted in media, and filter sterilized (which causes about a 20% loss of the vitamin [32]), and added to culture at a concentration of 1 μg/ml. Glutathione ethyl ester was dissolved in media and added to culture at a concentration of 250 μmol/l. Antimycin A (Sigma Chemicals) made 2.5 mmol/l in ethanol was added to culture at 10 μmol/l. Ethanol alone had no effect on *Pax-3* expression (data not shown). Following culture for 18 h, media were aspirated, and cells were solubilized in 0.5 ml UltraSpec (Biotecs, Friendswood, Tex., USA). Pax-3 mRNA was assayed by relative quantitative RT-PCR.

P19 Cell Culture. P19 embryonal carcinoma cells were cultured in DMEM with 25 mmol/l glucose (GIBCO-Life Technologies, Grand Island, N.Y., USA) plus 10% foetal calf serum (Sigma Chemicals). Cultures were grown as uninduced cultures or induced to differentiate into *Pax-3*-expressing cells with 0.3 μmol/l retinoic acid (RA) (Sigma Chemicals) [33, 38]. Antimycin A was added to cultures at indicated concentrations. Cells were harvested after 48 h of culture and Pax-3 mRNA was assayed by RT-PCR. DNA fragmentation was assayed [34] and electrophoresed alongside a 1 kb DNA molecular weight ladder (GIBCO-Life Technologies).

Relative quantitative (Real Time) RT-PCR of Pax-3 mRNA. RNA was prepared and 100 ng aliquots were reverse transcribed in quadruplicate [24]. One-tenth of each RT reaction was used as template for PCR using a Perkin Elmer 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, Calif., USA) according to the manufacturer's recommendations. PCR was performed using 0.5 μmol/l of forward and reverse primers (AAAAGGCTAAACACAGCATCGAT and TCGGAGCCTTCATCTGACTGA respectively), and 0.25 μmol/l of probe (CATCCTGAGTGAGCGAGCCTC-

TG-CA). Ribosomal RNA did not change relative to total RNA in all treatment groups and was assayed as an internal control, using rRNA Pre-Developed TaqMan Assay Reagents (PE Biosystems) according to the manufacturer's recommendations. Pax-3 PCR product was normalized to the rRNA PCR product and was expressed relative to that of embryos from one of the control pregnancies.

MDA Analyses. Immediately after the animals were killed, embryo decidua were placed on dry ice. Tissues were homogenized in 10 mmol/l Tris containing 50 mmol/l butylated hydroxy toluene (BHT) and aliquots were saved for protein assay by the Bradford method (BioRad, Hercules, Calif., USA). Lipid peroxidation was assessed by measuring malondialdehyde (MDA), an end-product of fatty acid peroxidation which can be used to estimate the extent of lipid peroxidation [35]. Briefly, 0.2 ml protein extracts were diluted in 0.8 ml PBS plus 0.025 ml BHT (50 mmol/l) and reacted with 0.5 ml of 30% thiobarbituric acid (TBA). The concentration of MDA-TBA complex was assessed by HPLC using an ion exclusion reverse phase Shodex KC-811 column (Waters) and 0.1% H_3PO_3 in water solvent. Variation in assay of MDA of the same sample on different days was less than 7%.

Statistical Analyses. Data were analyzed by 1-Way Analysis of Variance and Neuman Keuls post hoc test, using Prism 3 software (GraphPad Software, San Diego, Calif., USA). A p value less than 0.05 was considered to be statistically significant.

Results

α-tocopherol prevents the decrease in Pax-3 expression and the increase in NTD caused by maternal diabetes. To test the effect of reducing oxidative stress in embryos of diabetic mice, both diabetic and non-diabetic mice were fed control chow, or α-tocopherol-supplemented chow (which increased the dietary delivery of α-tocopherol 21-fold) beginning on day 0.5 of pregnancy. Blood glucose was increased in diabetic mice; α-tocopherol had no effect on glycaemia in either non-diabetic or diabetic mice (Table 1). Relative oxidant exposure in the intrauterine environment was estimated by assay of decidual malondialdehyde (MDA) on day 8.5, the day on which *Pax-3* expression and neural tube fusion begins. MDA was increased 1.6-fold by maternal diabetes (p=0.03), and this increase was prevented by α-tocopherol administration.

Pax-3 mRNA in embryonic day 8.5 embryos was assayed by relative quantitative RT-PCR. Maternal diabetes inhibited Pax-3 mRNA almost nine-fold (Fig. 1A; p<0.01), and this decrease was suppressed by α-tocopherol. Embryo size, somite number, and developmental morphology were the same in all treatment groups, indicating that the reduced expression of *Pax-3* in embryos of diabetic mice was not the result of growth or developmental delay (data not shown). There was an inverse relationship between reduced expression of *Pax-3* in embryos obtained on day 8.5 and NTD in embryos obtained on day 10.5 (Fig. 1B). NTD were increased in embryos of diabetic mice

Table 1. Effects of maternal diabetes and α-tocopherol on maternal blood glucose and embryo (decidual) MDA concentration

	Blood glucose (mmol/l)	MDA (nmol/mg)
Non-diabetic, control chow (4)	6.4±0.15	0.165±0.017
Diabetic, control chow (5)	20.0±1.68*	0.266±0.034**
Non-diabetic, α-t chow (5)	7.0±0.18	0.167±0.045
Diabetic, α-t chow (6)	15.9±3.11**	0.177±0.034

Number of pregnancies per treatment group is shown in parentheses. Each pregnancy contained 9-15 decidua.
Values are means ± SEM
α-t=α-tocopherol
* p<0.01 vs. Non-diabetic, control
** p<0.05 vs. Non-diabetic, control

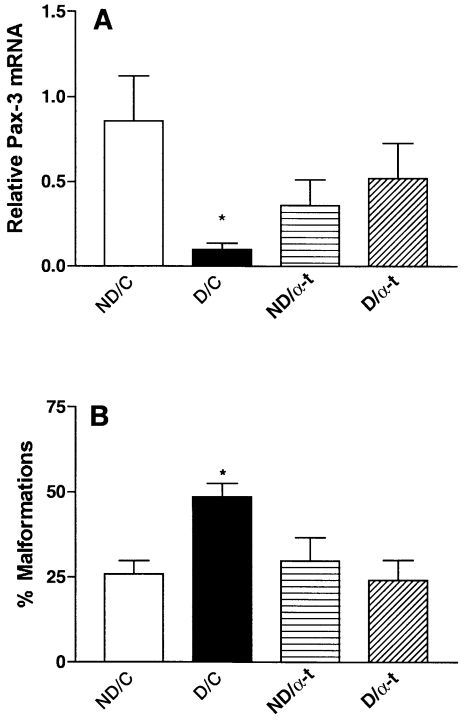

Fig. 1. (**A**) Relative quantitative RT-PCR of Pax-3 mRNA from day 8.5 embryos from pregnancies. ND, non-diabetic; D, diabetic; C, control diet; α-t, α-tocopherol-supplemented chow. *p<0.01 vs. ND/C, ND/α-t, D/α-t; differences between ND/C, ND/α-t, and D/α-t, were not significant. Nine to seventeen embryos were recovered from each pregnancy and were pooled for RT-PCR assay. n refers to the number of pregnancies per treatment group. ND/C (n=6); D/C (n=4); ND/α-t (n=5); D/α-t (n=4). (**B**) Percent malformation of embryos. *p<0.01 vs. non-diabetic/control and diabetic/α-tocopherol-supplemented chow; p<0.05 vs. non-diabetic/α-tocopherol-supplemented chow. Values represent mean malformation rate per pregnancy of 5 to 12 embryos ± SEM. ND/C (n=5); D/C (n=6); ND/α-t (n=4); D/α-t (n=6). It should be noted that 20 to 25% NTD in ND pregnancies of ICR mice is a consistent finding in our laboratory [24, 25] and probably is due to the large number of implanted embryos which are reabsorbed during later foetal development in an outbred strain

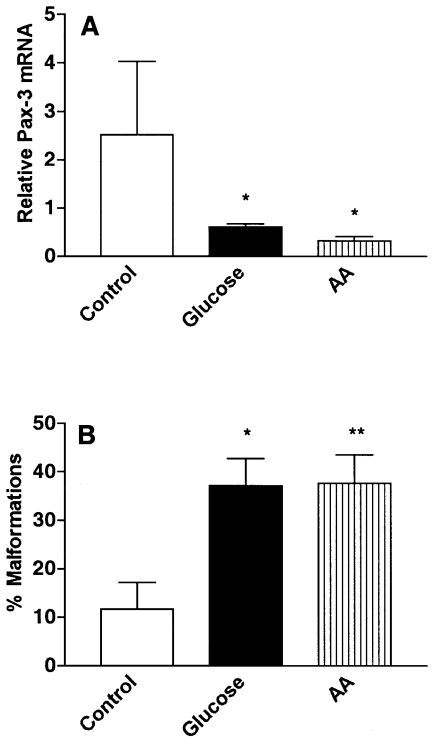

Fig. 2. (**A**) Relative quantitative RT-PCR of Pax-3 mRNA from day 8.5 embryos whose mothers had been injected on day 7.5 with propylene glycol (Control, n=3), glucose (n=3), or antimycin A (AA, n=5). Mean blood glucose concentrations on day 7.5 were 7.2±0.2, 16.9±0.3, and 7.3±0.2 mmol/l for control, glucose, and AA pregnancies, respectively. *p<0.05 vs. control. (**B**) Percent malformation of day 10.5 embryos whose mothers had been injected on day 7.5 with propylene glycol (Control, n=7), glucose (n=7), or antimycin A (AA, n=11). Mean blood glucose concentrations on day 7.5 were 7.3±0.3, 17.4±0.9, and 7.7±0.2 mmol/l for control, glucose, and AA pregnancies, respectively. *p<0.01 vs. control; **p<0.05 vs. control

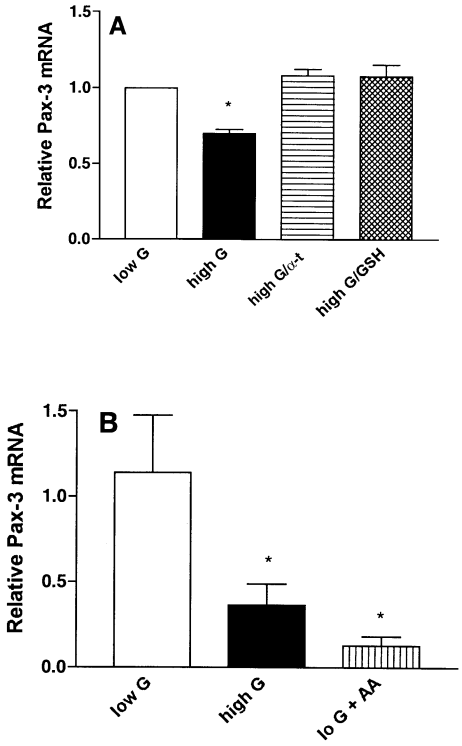

Fig. 3. (**A**) Pax-3 mRNA from primary embryo culture in low glucose (G) (7.5 mmol/l) or high G (25 mmol/l), with or without α-tocopherol or glutathione ethyl ester. *p<0.01 vs. low G. α-t, α-tocopherol; GSH, glutathione ethyl ester. (**B**) Pax-3 mRNA from primary embryo culture in low or high glucose, or low glucose with antimycin A. *p<0.05 vs. low G. AA, antimycin A

(p<0.005), and α-tocopherol suppressed the diabetes-induced NTD.

Induction of oxidative stress with antimycin A replicates the effects of maternal diabetes on Pax-3 expression and NTD. Previously, we showed that maternal hyperglycaemia on day 7.5 alone is sufficient to lead to reduced expression of Pax-3 on day 8.5 and increased NTD on day 10.5. To test whether oxidative stress induced on day 7.5 is sufficient to inhibit Pax-3 expression and to induce NTD, antimycin A was administered to pregnant mice in a single injection on day 7.5. Antimycin A is a complex III electron transport inhibitor which increases mitochondrial production of superoxide and hydrogen peroxide. A single injection of antimycin A inhibited Pax-3 expression by eight-fold (p<0.05), similar to the effect of inducing maternal hyperglycaemia with glucose injection (Fig. 2A). The decrease in Pax-3 expression on day 8.5 was correlated with increased NTD on day 10.5, as NTD were increased more than three-fold by antimycin A (p<0.05) or glucose injection (p<0.05) (Fig. 2B).

Oxidants inhibit Pax-3 expression by primary embryo culture. Further evidence that oxidative stress inhibits expression of *Pax-3* was obtained using primary culture of tissues obtained from neurulating mouse embryos. Pax-3 mRNA was reduced by culture in high (25 mmol/l) glucose-containing media compared to culture in low (7.5 mmol/l) glucose-containing media (p<0.01). α-tocopherol, as well as the antioxidant, glutathione (GSH) ethyl ester, each prevented the inhibition of Pax-3 mRNA by high glucose (Fig. 3A). Conversely, inducing oxidative stress by culture in media containing antimycin A inhibited *Pax-3* expression by primary mouse embryo culture (p<0.05) (Fig. 3B). This indicates that oxidants generated within the embryo itself are sufficient to inhibit *Pax-3* expression. Moreover, while oxidants could disturb maternal physiology or the development or function of extraembryonic membranes, these effects do not provide the sole basis for the adverse effects of oxidative stress on embryo gene expression.

Oxidative stress inhibits Pax-3 expression in a cell culture model of differentiating neuroepithelium. As an additional test of inhibition of *Pax-3* expression by oxidative stress, the effects of antimycin A on *Pax-3*

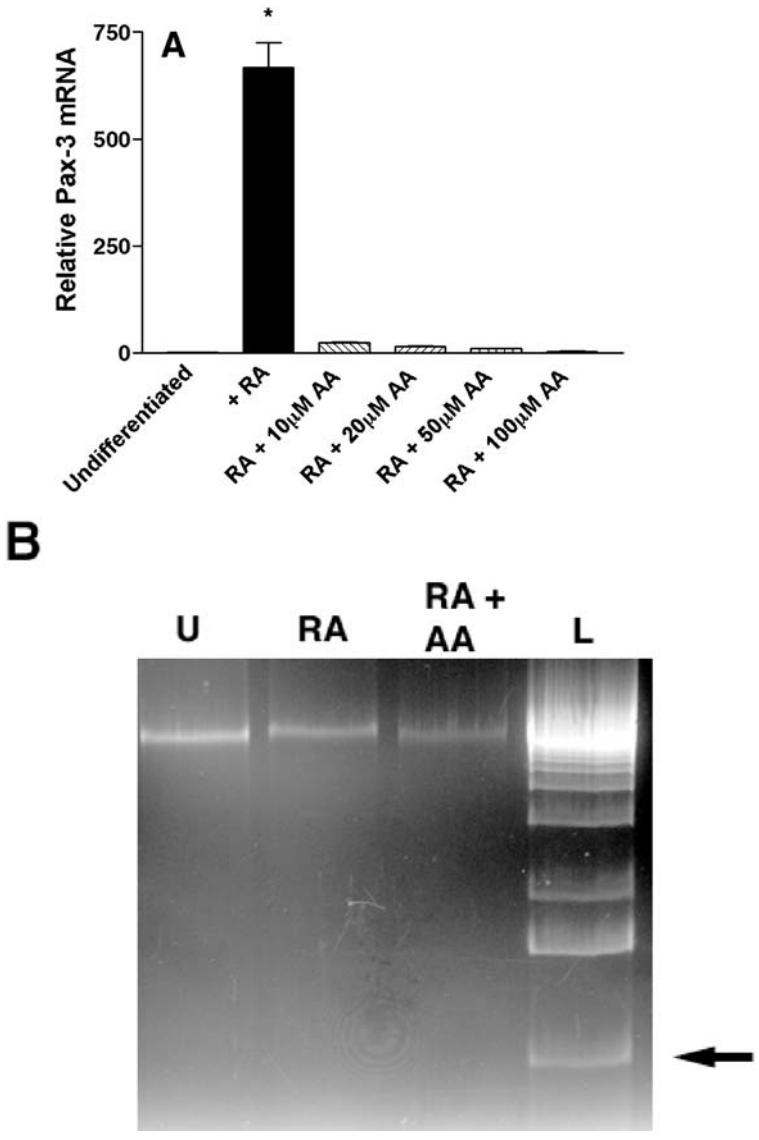

Fig. 4. (**A**) Pax-3 mRNA from P19 cells that were uninduced (U) or induced to differentiate with retinoic acid (RA), without or with antimycin A (AA). Antimycin A was added at indicated concentrations (10–100 μmol/l) from a stock solution of 2.5 mmol/l made up in ethanol. Ethanol alone had no effect on *Pax-3* expression (data not shown). *$p<0.001$ vs. undifferentiated or differentiated plus antimycin A. (**B**) Electrophoretic analysis of DNA from undifferentiated P19 cells (U), or P19 cells induced with retinoic acid (RA), with or without antimycin A (AA). L, 1 kb DNA ladder. Arrow indicates the position of the 1 kb marker

expression by P19 embryonal carcinoma cells were examined. P19 cells can be induced to differentiate into a neuroectodermal phenotype which expresses *Pax-3* with retinoic acid (RA). In addition, P19 cells can be grown in quantity, making it possible to test whether the inhibition of *Pax-3* occurs as a result of DNA damage. RA induced Pax-3 mRNA almost 700-fold ($p<0.001$), and all concentrations of antimycin A tested (10–100 μmol/l) inhibited Pax-3 mRNA induction (Fig. 4A). Antimycin A did not induce DNA strand breaks, an early sign of cells undergoing apoptosis, indicating that the oxidant effects were transcriptional, and were not due to gross genotoxic or cytotoxic effects (Fig. 4B).

Discussion

Numerous studies have shown that antioxidants can prevent the developmental defects caused by maternal diabetes in experimental animals [14, 18, 19, 20, 21, 22, 23]. Interestingly, vitamin E was first identified as an essential nutrient for prenatal (but not postnatal) life [39, 40]. Later, it was shown that vitamin E is required for formation of the neural tube by virtue of its antioxidant properties [41]. This indicates that embryonic development is exquisitely sensitive to oxidants generated even during normal metabolism, perhaps because of the immaturity of the free radical scavenging pathways [16, 42, 43], and that the increased fuel metabolism in embryos of diabetic mothers generates oxidants which increase the need for free radical scav-

enging in order to prevent the developmental defects resulting from oxidative stress. The mechanisms by which oxidants can disturb development have not been understood. Our data suggest that oxidants disturb the expression of genes which direct developmental programs. Ultimately, cell death could be the cause of maldevelopment, but this might be secondary to failure of a developmental program.

In general, the teratogenic effects of oxidative stress have been attributed to cytotoxic or genotoxic processes. However, if oxidants cause embryonic defects by inducing cell death, then there must be some mechanism to explain how only some cells are killed while others are spared, despite equivalent exposure of all embryonic cells to the source of the oxidative stress. It has been noted that if embryonic cell death induced by teratogens (including oxidative stress) were severe, then the whole embryo would die, while comparatively mild teratogen exposure would kill only some cells [44]. In attempting to explain the localized nature of teratogen-induced malformations, it has been proposed that cells within the vicinity (temporally and spatially) of cells which die as part of a normal developmental program could be particularly vulnerable to teratogen-induced cell death [45]. However, these explanations are not consistent with the NTD induced by oxidative stress associated with diabetes, since the neuroepithelium does not undergo apoptosis prior to fusion of the neural tube.

The data here purport that oxidants can be teratogenic simply by interfering with expression of genes which control essential developmental processes. Using *Pax-3* as a model, increased oxidant exposure during a critical window of time, day 7.5, will inhibit its expression on day 8.5. Currently, we can only speculate how this occurs. Since transcription factors can be regulated by cellular redox status, and activity of growth factor and cell cycle signals can be regulated by protein glutathiolation [31, 46, 45, 46, 47, 48], signals which are needed to induce *Pax-3* gene expression could be affected.

At this time, we cannot determine the degree to which *Pax-3* expression must be suppressed in individual embryos in order to result in NTD. This is because Pax-3 mRNA is assayed in embryos recovered on day 8.5, and NTD must be scored in different embryos recovered on day 10.5. However, while NTD occur with 100% penetrance in *Sp/Sp* embryos (in which Pax-3 production is 0% that of wild type), neural tube development is normal in *Sp/+* embryos (in which Pax-3 production is 50% that of wild type). Therefore, there must be a critical threshold (greater than 0% but less than 50% wild type expression) that is sufficient for normal neural tube closure. Indeed, it would be interesting if a technology is developed in the future that would allow assay of *Pax-3* expression in individual embryos on day 8.5, and assess if they display NTD on day 10.5, in order to determine where, relative to wild type expression, the critical threshold lies.

We recently showed that Pax-3 down regulates p53 protein, and that NTD can be prevented in Pax-3-deficient *Sp/Sp* embryos by p53 deficiency [49]. Thus, apoptosis is ultimately responsible for the NTD associated with Pax-3 deficiency, but only because Pax-3 is needed to inhibit p53-dependent apoptosis until fusion of the neural tube is complete. It remains to be determined what induces p53-dependent apoptosis in the neural tube, thereby making *Pax-3* expression necessary. An intriguing theoretical explanation is that, during normal development, oxidants would naturally increase as the embryo begins to increasing rely on oxidative metabolism, rather than predominantly anaerobic metabolism, at this stage of development [50]. In fact, some amount of oxidant production might have a positive effect on *Pax-3* expression. Indeed, it should be noted that Pax-3 mRNA was slightly reduced in non-diabetic pregnancies treated with α-tocopherol. While this might simply be due to the small sample size, it could instead indicate that some oxidant exposure is needed for optimal induction of *Pax-3*. (It should be noted that the difference in Pax-3 mRNA between non-diabetic pregnancies that were or were not treated with α-tocopherol was neither statistically nor biologically significant, as there was no difference in the rate of NTD.) Nevertheless, because oxidative stress can activate p53-dependent apoptosis [51, 52], neuroepithelium, which develops at the same time that oxidative metabolism increases, could be at particular risk for p53-dependent apoptosis. Thus, Pax-3 might be required to override p53-dependent apoptosis in order to allow neural tube formation to proceed. If our model is correct, then embryos of diabetic mothers could be particularly vulnerable to NTD ultimately caused by p53-dependent apoptosis. Hyperglycaemia-induced oxidative stress would activate p53, and by suppressing expression of Pax-3, down regulation of p53 would be impaired. In preliminary experiments, we have found that p53 deficiency provides protection from NTD in embryos of diabetic mice (unpublished results). In the future, it will be important to understand the interaction of oxidant activation of p53 and inhibition of Pax-3 during the aetiology of NTD.

Oxidant inhibition of *Pax-3* expression might explain NTD from a variety of causes in addition to diabetic pregnancy. Of note, folic acid supplementation seems to prevent NTD in some susceptible embryos by blocking accumulation of homocysteine, a cellular oxidant [53, 54, 55]. Many drugs that cause neural tube, and other, defects, including thalidomide [56], phenytoin [56, 57], environmental aryl hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [58], and ionizing radiation [59] induce oxidative stress. Myelomeningocele has been linked with reduced glutathione peroxidase activity apparently resulting from a genetic

polymorphism [60]. Finally, maternal obesity, which could increase embryo oxidant exposure due to maternal glucose intolerance, has been found to increase the relative risk for NTD [61, 62].

In conclusion, increased oxidant exposure during embryogenesis could lead to congenital defects by disturbing the expression of genes which control essential developmental processes. Since *Pax-3*, whose expression is inhibited by oxidative stress, is essential for neural tube development, reduced *Pax-3* expression in the neural tube could provide a unifying explanation for NTD caused by maternal diabetes, as well as other sources of oxidative stress.

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