considered to be less differentiated[12]. Few mast cells in the membranes examined had discharged their granular contents. Accounts of when mast cells first develop are conflicting[13]; however this report of the presence of mast cells at 3 days in the chick yolk-sac vasculature is the earliest at which mast cells have been identified. Recently mast cell precursors have been discovered in the early mouse yolk sac at 9.5 days (equivalent to 3 days of chick embryo development), but the method employed was not able to ascertain at what time the precursors gave rise to characteristic mast cells[14].

The presence of mast cells on the yolk-sac vasculature is significant because it shows that heparin-producing cells are present during a phase of extensive capillary growth in a developing embryonic system. Thus it appears, that the mechanism proposed for tumor angiogenesis, that is, heparin produced by mast cells enhances the directed migration of capillary endothelial cells towards the tumor[15], could also apply to embryonic angiogenesis. Such a unifying mechanism would account for the similar effects of protamine on both tumor and embryonic angiogenesis.

1 The work is supported by The Wellcome Trust.
2 Ausprunk, D.H., Knighton, D.R., and Folkman, J., Devl Biol. *38* (1974) 237.
3 Kumar, S., West, D., Shahabuddin, S., Arnold, F., Haboubi, N., Reid, H., and Carr, T., Lancet *2* (1983) 364.
4 Auerbach, R., Kubai, L., and Sidky, Y., Cancer Res. *36* (1976) 3435.
5 Phillips, P.J., Steward, J.K., and Kumar, S., Int. J. Cancer *17* (1976) 549.
6 Taylor, S., and Folkman, J., Nature *297* (1982) 307.
7 Kessler, D.A., Langer, R.S., Pless, N.A., and Folkman, J., Int. J. Cancer *18* (1976) 703.
8 Azizkhan, R.G., Azizkhan, J.C., Zetter, B.R., and Folkman, J., J. exp. Med. *152* (1980) 931.
9 Romanoff, A.L., The Avian Embryo. Macmillan, New York 1960.
10 Summerbell, D., and Hornbruch, A., in: Culture Techniques-Applicability for Studies on Prenatal Differentiation and Toxicity. Eds D. Neubert and H.J. Merker. Walter de Gruyter, Berlin 1981.
11 Padawar, J., J. Histochem. Cytochem. *7* (1959) 352.
12 Combs, J.W., J. Cell Biol. *31* (1966) 563.
13 Seyle, H., The Mast Cells. Butterworth, London 1965.
14 Sonada, T., Gayashi, C., and Hitamura, Y., Devl Biol. *97* (1983) 89.
15 Folkman, J., Taylor, S., and Spillberg, C., in: Development of the Vascular System, p. 132. Ciba Foundation Symposium No. 100, Pitman, London 1983.

0014-4754/85/020269-03$1.50 + 0.20/0

Evidence for reopening of the cranial neural tube in mouse embryos treated with cadmium chloride

B.P. Schmid[1,3], J. Kao[2] and E. Goulding

Laboratory of Reproduction and Developmental Toxicity, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park (North Carolina 27709, USA), 26 August 1983

Summary. Use of the whole-embryo culture technique resulted in experimental evidence that the pathogenesis of exencephaly in mouse embryos after cadmium chloride treatment results from reopening of the cranial neural tube.
Key words. Mouse embryos; neural tube closure; cadmium chloride; embryo cultures; exencephaly; teratogenicity.

Neurulation is one of the earliest and most important processes that developing embryos undergo during the organ formation phase. This process is a very complex one, during which various morphogenetic events take place simultaneously at an extremely rapid rate. In the mouse, for example, the central nervous system (CNS) primordium is established within about a two-day period.

It is known that chemicals can cause CNS abnormalities during embryonic and fetal development. The most common CNS defects are anencephaly in humans[4,5] and exencephaly in experimental animals (for review, see Lemire et al.[6]). The pathogenesis of these anomalies is as yet unknown, as is whether they are due to non-closure or reopening of the neural tube. This study reports the effects of cadmium chloride ($CdCl_2$) on the neural tube closure of mouse embryos. $CdCl_2$ was selected for this study, as this chemical was shown to produce high incidences of exencephaly in term mouse fetuses when administered to pregnant mice during organogenesis[8], as well as to cause changes in the neuroepithelium during the process of neural tube closure[9]. $CdCl_2$ administration to pregnant rats at various intervals after copulation has shown the greatest percentage of accumulation in embryos which were at the early phase of organogenesis[10].

Investigations were carried out with the aid of the postimplantation embryo culture technique[7]. This method not only provides access to embryos at the early stages of organogenesis but also enables direct extracorporeal observation and manipulation of the embryos.

Material and methods. Medium preparation. The medium consisted of 100% male rat serum. Blood for the serum was taken from the dorsal aortas of CD-rats (Charles River Laboratories, Wilmington, MA), and centrifuged immediately. The serum was then decanted and stored at −20 °C. All sera were carefully thawed and heat-inactivated at 56 °C for 40 min prior to use. *Culture conditions.* Early organogenesis CD-1 mouse conceptuses (Charles River Laboratories, Wilmington, MA) were explanted on day 8 (plug day = 0) and the maternal decidua and Reichert's membrane removed. The embryos (3–5 somites) within their yolk-sacs and amnions were cultured at 37.5 °C for

Effects of cadmium chloride on mouse neural tube tube development in vitro observed after a) 24 h and b) 48 h exposure periods

	Cadmium chloride ($\times 10^{-6}$ M)	No. of embryos treated	Embryos showing open neural tubes	Mean morphological score
a)	0.0	25	0 (0)	28.2 ± 0.1
	1.1	14	0 (0)	26.1 ± 0.1
	1.3	14	0 (0)	26.3 ± 0.2
	1.6	6	0 (0)	26.2 ± 0.2
b)	0.0	82	0 (0)	43.3 ± 0.2
	1.1	13	4 (30.8)	39.5 ± 1.3
	1.3	10	2 (20.0)	42.6 ± 0.4
	1.6	14	7 (50.0)	37.1 ± 1.9

Numbers in parentheses: percentages.

24 h or 48 h. Various concentrations of $CdCl_2$, or the distilled water vehicle, were present during the entire culture period. Two embryos per bottle were cultured in 5 ml of serum, the bottles were rotated at 11 rpm. Gassing conditions were as described previously[11]. All operations were carried out aseptically, and no antibiotics were used. At the end of the culture period the embryos were dissected from the yolk-sac and amnion. Embryos which had fully rotated, and had a heart beat and fully developed yolk-sac blood circulation, were evaluated according to a morphological scoring system[12]. This scoring system also included a check for neural tube defects.

Results and discussion. Tissue of the early somite stage embryos (fig. 1) cultured in control serum for 24 h became extensively differentiated into the primordia of neural, sensory, cardiac, circulatory and hepatic tissues. At this stage the embryos contained about 21 somites, were ventrally curved and the neural regions were closed (table, a). Embryos cultured in the presence of $CdCl_2$ at concentrations as high as 1.6 µM were comparable in morphology and size to the control embryos, and had comparable differentiation scores (table, a).

A concentration-dependent variety of dysmorphogenic effects was, however, observed in embryos cultured in the presence of $CdCl_2$ for 48 h. Non-closure of the cranial neural tube region was highly evident (fig. 2, table, b), indicating that the in vitro system responded similarly to in vivo $CdCl_2$ treatment. The abnormality was often associated with irregular fusion of the telencephalic and mesencephalic regions, and with stunted telencephalic hemispheres. Other differentiation parameters were not significantly affected, as the morphological scores were comparable to those of the controls. No apparent adverse effects were observed in the 82 control embryos also maintained for 48 h in vitro.

It has been shown that treatment of day-8 mouse embryos in utero with 4 mg/kg $CdCl_2$ resulted in histopathological changes of the neuroepithelium already 8 h after treatment[9]. The effects observed were dark-staining bodies, and were interpreted as autophagic vacuoles, or cytolysosomes. These findings indicate that alterations on a cellular level can occur before neural tube closure takes place. Nevertheless, as shown in the present study, the cranial neural tube is able to close. Reopening of the cranial neural tube, on other hand, may also be the result of damage initiated in the neuroepithelium on days 8–9 by $CdCl_2$.

Most investigators have postulated from observations of experimentally produced exencephaly in animals that the origin of exencephaly is in most cases due to a failure of the rostral part of the neural tube to close. These investigations were carried out in whole-animal systems, which have the disadvantage that direct access to the embryo is impossible. Moreover, intra- and interuterine variability of the developmental embryonic stages is known to be great. In mice, for example, intrauterine differences in embryonic development have been shown to be four Theiler stages[15], which corresponds to about a 2-day period. Our findings indicate that a 24 h $CdCl_2$ exposure period of mouse embryos in vitro resulted in normal embryos, whereas 48 h $CdCl_2$ treatment caused a high incidence of cranial neural tube defects. This suggests a reopening of the neural tube in this region within a few hours. Basic mechanistic studies on this type of anomaly should therefore be carried out under defined conditions, e.g. by the use of the postimplantation embryo culture system.

Siegers and his collaborators recently reported that $CdCl_2$ concentrations measured in the amniotic fluid of smoking pregnant women averaged around 8 ng/ml[14]. This concentration is about $^1/_{25}$ of the lowest concentration causing exencephalies in our in vitro experiments (1.1 µM). These low concentration levels found in the immediate environment of the developing human embryo could explain the fact that, though the teratogenicity of $CdCl_2$ in experimental animals is well documented, embryotoxic effects could not be found in the human offspring[13].

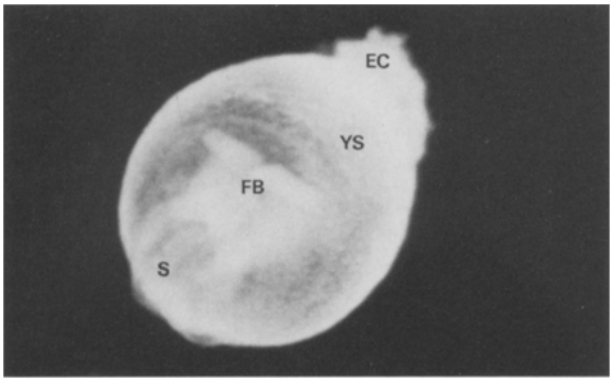

Figure 1. Day-8 mouse conceptus after dissection of the maternal compartment (Theiler stage 12). Forehead region (FB) and somites (S) are visible through the yolk-sac membrane (YS); EC: ectoplacental cone (6.5:1).

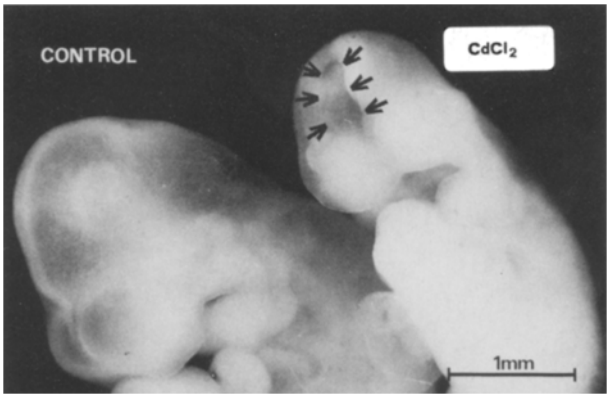

Figure 2. Representative photomicrograph of mouse embryos cultured for 48 h in either the absence (control) or presence of 1.6 µM cadmium chloride ($CdCl_2$). The open neural region in the mesen-/telencephalic area is indicated by arrows.

1 Present address: Toxicology Dept., ZYMA SA, CH-1260 Nyon, Switzerland.
2 Present address: Oak Ridge National Laboratory, P.O. Box Y, Oak Ridge, Tennessee 37830, USA.
3 Address to which reprint requests should be sent.

4 Holmberg, P.C., and Nurminen, M., Am. J. ind. Med. *1* (1980) 167.
5 Sever, L.E., Teratology *25* (1982) 323.
6 Lemire, R.J., Beckwith, J.B., and Warkany, J., eds, Anencephaly, p. 85. Raven Press, New York 1978.
7 New, D.A.T., Biol. Rev. *53* (1978) 81.
8 Kao, J., Schmid, B., Brown, N.A., Goulding, E., and Fabro, S., Fedn Proc. *39* (1980) 39.
9 Webster, W.S., and Messerle, K., Teratology *21* (1980) 79.
10 Ahokas, R.A., and Dilts, P.V., Am. J. Obstet. Gynec. *135* (1979) 219.
11 Schmid, B.P., Trippmacher, A., and Bianchi, A., Toxicology *25* (1982) 53.
12 Brown, N.A., and Fabro, S., Teratology *24* (1981) 65.
13 Degraeve, N., Mutat. Res. *86* (1981) 115.
14 Siegers, C.-P., Jungblut, J.R., Klink, F., and Oberheuser, F., Toxic. Lett. *19* (1983) 327.
15 Juurlink, B.H.J., and Federoff, S., In Vitro *15* (1979) 86.

0014-4754/85/020271-02$1.50 + 0.20/0