Int. J. Dev. Biol. 44: 161-175 (2000)

Studies of the mechanism of amniotic sac puncture-induced limb abnormalities in mice

MATTHEW H. KAUFMAN* and HAN HSIN CHANG

Department of Anatomy, University Medical School, Edinburgh, United Kingdom

ABSTRACT The principal advantage of chorionic villus sampling (cvs) over amniocentesis for the determination of the genetic constitution of the embryo is that it may be undertaken earlier in pregnancy. If carried out too early in pregnancy, it has the risk of inducing craniofacial and limb abnormalities, a condition termed the oromandibulofacial limb hypogenesis (OMFL) syndrome in genetically normal infants. It is believed that the defects observed have a vascular origin, possibly due to anoxia of tissues due to fetal blood loss or thrombus formation at the site of biopsy with distal embolization. We believe that this does not adequately explain the findings from the experimental animal literature involving amniotic sac puncture (ASP). Based on these experimental findings, we have hypothesised that (i) the defects observed following cvs may result from the consequences of oligohydramnios following the inadvertent puncturing of the amniotic sac during this procedure, and (ii) that cleft palate and the postural limb defects observed (e.g., clubfoot and clubhand) are secondary to embryonic/fetal compression. Our experimental studies shed new light on the mechanism of induction of the limb defects seen, but particularly syndactyly. Evidence of hypoperfusion of the peripheral part of the developing limb bud is observed, which interferes with apoptosis that occurs in the digital interzones, or induces an abnormal degree of cellular proliferation and/or tissue regeneration in these sites, possibly because of over-expression of critical genes involved in limb pattern specification. Cleft palate, tail abnormalities and abnormalities of sternal ossification are also observed in our model.

KEY WORDS: *chorionic villus sampling, oligohydramnios, tissue hypoperfusion, limb and craniofacial abnormalities, embryo compression*

Early reservations concerning the safety of amniocentesis

Shortly after the first reports of the successful use of amniocentesis for the prenatal diagnosis of human fetal sex from the analysis of desquamated fetal cells present in the amniotic fluid isolated between 8 weeks of gestation and full term (Dewhurst, 1956; Makowski *et al.*, 1956; Sachs *et al.*, 1956), reservations were expressed with regard to the safety of the procedure based on the results of experimental animal studies. It should be noted that even in Dewhurst's (1956) report, attention was drawn to the risks involved in undertaking amniocentesis late in gestation, because of the possibility of causing injury to the fetus or placenta or even to the uterus, and additionally inducing the premature onset of labour. Indeed it was for these reasons that this author felt that the risks involved, however slight, were too great, and concluded that "*the procedure was unsuitable for general use to discover a child's sex which would soon be known for certain.*" In contemporary experimental studies, animal models were described which it was believed shed light on the possible dangers of amniocentesis, particularly when this procedure was carried out at much earlier stages of gestation, at times approximately equivalent to the latter part of the first trimester of pregnancy.

In the first of these experimental studies, Trasler *et al.* (1956) reported that experimentally-induced amniotic fluid leakage caused teratological abnormalities. The authors performed laparotomies on pregnant female mice, and inserted a needle through the wall of one uterine horn into the individual amniotic sacs, inducing the uncontrolled leakage of amniotic fluid; the implantation sites in the contralateral uterine horn acted as non-injected (or 'internal') controls. The females were autopsied on day 18.5 of pregnancy, just before term, and the gross morphological features of the control and experimental embryos were studied. Of the 14 treated females, 6 resorbed their litters. In the remaining 8 litters, 10 out of 17 treated embryos that survived had cleft palates, whereas the palates of the 15 control embryos were all closed. The difference was highly significant. The authors concluded that ... "*These cleft palates appear to have resulted from a loss of amniotic fluid, which constricted the embryo, pushing the head down on the chest and*

***Address for reprints:** Department of Anatomy, University Medical School, Teviot Place, Edinburgh EH8 9AG, United Kingdom. FAX: 0131-650-6545. e-mail: M.Kaufman@ed.ac.uk

0214-6282/2000/$20.00

Printed in Spain

forcing the lower jaw upward. Thus the tongue was forced between the palatine shelves, which therefore could not fuse. Our results with mice suggest that there may be a definite risk to the baby in inserting a needle into the amniotic sac in human beings, especially during the early stages of pregnancy when there is a danger of inducing abnormalities in the developing embryo." It should be noted that the stage of development of the *mouse* embryos at the time of experimental intervention was equivalent to about 50-51 to 56-57 days post-ovulation, or Carnegie Stages 20-23 of *human* embryonic development (O'Rahilly and Müller, 1987; Kaufman, 1992).

In subsequent studies by Walker (1959), attempts were made to determine the underlying mechanism of induction of the palatal defect observed, and the findings led the author to the hypothesis that the uterine muscle and fetal membranes could compress the embryo and cause cleft palate when the hydrostatic pressure in the amniotic sac is reduced, and the uterus is thus allowed to compress the embryo. Additional studies revealed that if the experimental embryos were released from the constraints imposed by the uterus at the time of treatment, they were not noticeably compressed when examined within 1-2 days, though by full term mild signs of compression were present. The latter finding appeared to indicate that the extra-embryonic membranes could also have a slight constraining effect on the embryo. In addition to noting palatal defects, "*limbs twisted out of position*" were also observed in some instances.

The next important paper that describes experimental findings relevant to this topic, with the pregnant rat as the species studied, but using the same amniotic sac puncture system to that described by Trasler *et al.* (1956) was published by Poswillo (1966). He first drew attention to the fact that the type of palatal defect observed in these studies more closely resembled those found in humans than did the palatal defects produced by any other method previously used. He also emphasised that the likely teratogenic mechanism involved also occurred spontaneously in man to produce a similar type of defect (see below), and that the causal mechanism involved in producing the palatal defects in these cases also induced other associated malformations such as of the mandible and limbs. The findings from a more extensive study by the same author were published several years later (Poswillo, 1968).

As in the earlier mouse studies of Trasler *et al.* (1956), Poswillo carried out amniotic sac puncture (ASP) in rats (a) prior to the time of elevation of the palatal shelves. He additionally investigated the effect of this procedure carried out (b) during the period when the palatal shelves were in the process of elevating. He demonstrated, as had previous studies, that in all cases where a cleft palate was present, the tongue was found to be wedged between the palatal shelves; the mandible also showed evidence of compression, and its development was judged to be retarded with evidence of micrognathia (abnormally small size of the lower jaw). He concluded that "*the primary teratogenic mechanism was adduced to be craniocaudal compression resulting from an adverse change on fetal posture due to contraction of the amnion following leakage of amniotic fluid*."

All of the experimental embryos from series (b) showed evidence of malformations due to compression. Clefts of the palate were observed in 31/32 embryos, and the defects present varied from mild, with no tongue obstruction between the palatal shelves, to severe, with micrognathia and glossoptosis (downward displacement or retraction of the tongue); all embryos exhibited micrognathia to a greater or lesser degree. In addition, in 9/31 (29%) of these embryos, the limbs were compressed into abnormal positions, and often displayed deformities. The limb malformations observed varied from those believed to result from "ring constriction" (see below) to those in which parts were absent. In a subsequent report (Poswillo, 1968) the effect of ASP carried out after palatal shelf fusion was also described; while such embryos possessed an intact palate, they invariably displayed a severe degree of micrognathia.

Poswillo and Roy, (1965) and Poswillo (1966) noted micrognathia and glossoptosis in rat embryos with cleft palate following ASP, and suggested an analogy with what at the time was termed the human Pierre Robin syndrome (or 'sequence'; see Robin 1923, 1929; Jones, 1988) in which the triad of cleft palate, micrognathia and glossoptosis are observed, but occurs spontaneously and is of unknown aetiology; about 20% of these infants also displayed limb abnormalities (Routledge, 1960; Smith and Stowe, 1961). The current concept of the Pierre Robin sequence no longer includes limb abnormalities, so that the phenotype now exclusively relates to the craniofacial lesions indicated above, and some of the infants previously ascribed to this syndrome would now be reclassified into a number of other disorders. About 30% of infants previously recognised as having the Robin sequence would now, for example, be diagnosed as having the Stickler syndrome in which there is an association between ocular findings (e.g., severe myopia usually present before the age of 10, retinal detachment and/or cataracts), hearing loss and cleft palate (Gorlin *et al.*, 1990). Other disorders with some degree of overlap include Kniest dysplasia, SED congenita, and short stature, low nasal bridge, cleft palate and sensorineural hearing loss (Gorlin *et al.*, 1990).

This association between craniofacial and limb abnormalities has been a consistent finding in subsequent ASP studies in the rat (DeMyer and Baird, 1969; Love and Vickers, 1972; Singh and Singh, 1973; Singh *et al.*, 1974; Kino, 1975; Kennedy and Persaud, 1977; Houben, 1980, 1984; Houben and Huygens, 1987), though until relatively recently (see below) such a relationship had not been reported in the mouse.

While a considerable degree of unanimity exists concerning the mechanism(s) underlying the craniofacial lesions following ASP, namely that these probably have a postural basis, resulting from the compression of the embryo due to oligohydramnios, various hypotheses have been suggested to account for the *other* abnormalities commonly encountered following ASP. The mechanism of palate closure requires spontaneous muscular movement by the embryo in which extension of the neck occurs. This lifts the head away from the chest, thus permitting movements of the lower jaw and tongue (Walker, 1969). The postural hypothesis for the palatal defects seen following ASP contrasts with findings from other teratological approaches (e.g., Dostal and Jelinek, 1974) in which exposure of pregnant mice to certain substances on days 12-14 of pregnancy caused either the delayed *initiation* (exposure to 6-azauridine) or *progression* (exposure to cortisone) of horizontalization of the palatal processes and their abnormal narrowing once they had become horizontal.

While the most consistent postcranial abnormalities reported are of the limbs, defects of the tail and sternal ossification have also been reported. It is the consideration of the various possible mechanisms involved in the induction of abnormalities of these other systems that forms the basis of the rest of this review.

Clinical relevance of these studies

Before discussing the information that may be gained from the analysis of the various experimental animal models in which ASP has been employed, it is appropriate to briefly consider why there has been an upsurge of interest in this area of research. Before the technique of chorionic villus sampling *(cvs)* was introduced, the only method available that allowed access to embryo-derived material for diagnostic purposes was via the technique of amniocentesis. While it is technically possible to obtain a sample of amniotic fluid from about 8 weeks of gestation (i.e., 6 weeks post-fertilisation, see also below), amniocentesis is usually undertaken between about 12 and 16 weeks of pregnancy. In addition to providing cellular material suitable for genetic and cytogenetic analysis, it also provides a sample of amniotic fluid which may itself be analysed biochemically. From about 6-11 weeks of pregnancy, other invasive techniques have more recently been developed that allow small samples of extraembryonic tissue suitable for DNA or cytogenetic analysis to be isolated, namely via the technique of trans-cervical or trans-abdominal *cvs* (see Department of Obstetrics and Gynaecology, Tietung Hospital, 1975; Liu *et al.*, 1987; Lilford, 1990; Wapner, 1997).

Numerous clinical trials have been undertaken from the mid-1980s onwards to establish the advantages and/or disadvantages of *cvs* over amniocentesis. It is now generally accepted that the principal advantage of *cvs* over amniocentesis is that, if a genetic abnormality is diagnosed (Gosden, 1990; Lilford and Gosden, 1990), an even earlier and less traumatic termination of pregnancy may be performed should a termination be requested or considered appropriate. It only became apparent in the early 1990s that the principal *disadvantage* of *cvs* over amniocentesis is that if this procedure is undertaken *too early* in pregnancy (see below) it may, in a relatively small proportion of cases, lead to the birth of a genetically normal infant with craniofacial and/or limb abnormalities. It was also noted that there were significantly fewer surviving children in the *cvs* compared to the amniocentesis group; this difference reflected the significantly greater number of spontaneous fetal deaths before 28 weeks, more spontaneous miscarriages and more fetal deaths identified on ultrasound examination following *cvs* (MRC Working Party, 1991). Confined chorionic/placental mosaicism had also previously been recognised as an occasional complication in the interpreting of *cvs* findings (Kalousek *et al.*, 1987). While formerly the published data indicated that pregnancies in which *cvs* was performed were at greater risk of pregnancy loss than those in which amniocentesis was performed, it is now apparent that any differences between these two techniques in this regard is probably related to differences in the experience of the operators involved (Wapner, 1997).

Since the early 1990s, with the publication of the findings of an extensive MRC trial on the evaluation of *cvs* (MRC Working Party, 1991), concern has been expressed with regard to a possible association between *cvs* carried out between days 56-66 of gestation (as measured from time of last *normal* menstrual period, i.e., about 41-51 days post-coitum) and the birth of *genetically normal* infants with limb reduction defects, and orofacial and other congenital malformations. Many single and multiple case reports describing clinical experiences from around the world suddenly started to appear, principally in the pages of the *Lancet*, all of which described the occasional, but unexpected and consequently extremely disturbing, birth of genetically normal infants with craniofacial and/or limb abnormalities following *cvs* (see, for example, Hogge *et al.*, 1986; Planteydt *et al.*, 1986; Canadian Collaborative CVS-amniocentesis clinical trial group, 1989; Christiaens *et al.*, 1989; Rhoads *et al.*, 1989; Kaplan *et al.*, 1990; Editorial, 1991; Firth *et al.*, 1991a,b, 1994; Hsieh *et al.*, 1991, 1995; Mahoney, 1991; Mastroiacovo and Cavalcanti, 1991; Rodriguez and Palacios, 1991; Shepard *et al.*, 1991; Brambati *et al.*, 1992; Burton *et al.*, 1992, 1993; Froster and Baird, 1992; Kuliev *et al.*, 1992; Mastroiacovo *et al.*, 1992; Quintero *et al.*, 1992; Schloo *et al.*, 1992; Gruber and Burton, 1994; Mastroiacovo and Botto, 1994; Olney *et al.*, 1995; Botto *et al.*, 1996).

The *spontaneous* incidence of the syndrome complex with craniofacial and limb abnormalities similar to that observed in these infants is said to be in the region of 1: 175,000 livebirths (Froster-Iskenius and Baird, 1989) and is termed the oromandibulofacial limb hypogenesis (OMFL) syndrome; its reported occurrence following *cvs* has varied between 0.09% (Schloo *et al.*, 1992) and 1.7% (Firth *et al.,* 1991a).

It was generally considered that these abnormalities had a vascular basis (Report of NICHHD Workshop, 1993), this conclusion being largely influenced by the earlier experimental findings of Brent and co-workers (Brent and Franklin, 1960; Brent, 1990, 1993), since similar craniofacial defects had been induced following vascular disruption of the pregnant uterus in the rat, and limb and craniofacial defects had been induced following uterine trauma and clamping of the uterine arteries for between 14 and 90 min (Leist and Grauwiler, 1974; Webster *et al.*, 1987; see also Hoyme *et al.*, 1982). While it is most unlikely that the analogy between complete cessation of uterine blood flow and *cvs* is a direct one, haemorrhagic (ecchymotic) lesions involving the human limbs, scalp, face and thorax have been reported following experimentally-induced placental trauma (Quintero *et al.*, 1992). Using embryoscopy, they observed the surface features of a fetus at 9.5 menstrual weeks following transvaginal *cvs*; ultrasonography carried out during *cvs* displayed the development of a subchorionic haematoma. In a subsequent study, they performed embryoscopy before and after *cvs* carried out at between 8 and 12 weeks of pregnancy in 7 patients scheduled to have elective terminations of pregnancy, but observed no evidence of cutaneous haemorrhages. In the same cases, they also detached regions of the placenta with a blunt instrument, producing subchorionic haematomas, but again failed to observe limb lesions, and concluded that any relationship between placental vascular trauma and craniofacial and/or limb abnormalities is at most a tenuous one.

In a letter to the *Lancet*, Shepard *et al.* (1991) raised the possibility that inadvertent puncturing of the amniotic membranes during *cvs* could account for the limb abnormalities observed, extrapolating from the experimental ASP findings of Kennedy and Persaud (1977) who reported that amputation-type distal limb malformations were preceded or accompanied by vascular disruption presenting as haemorrhage or thrombosis. Shepard *et al.* hypothesised that ASP following *cvs* might possibly induce the formation of amniotic bands (these are fibrous bands passing from the fetus to the amnion of unknown aetiology, and it has been suggested that in some instances these may produce “ring constrictions” of the distal part of the limbs, leading to ischaemia and occasionally amputations), though they admitted that this was unlikely because no observations of such bands had been reported following *cvs* (the literature on the significance or otherwise of amniotic bands is extensive, and this was commonly cited as one

cause of congenital amputations [see, for example, Ballantyne (1902), and for more recent reviews, see Torpin (1968), and Hunter and Carpenter (1986)]; loss of amniotic fluid could result in compression and deformation of the embryo; thirdly, they hypothesised that there may occasionally be entrapment of the distal extremities of the limbs in the exocoelomic gel (located between the amnion and chorion, and said only to be present before day 63 of pregnancy). They suggested that ultrasonic evidence of limb entrapment immediately following *cvs* might be indicative of such an event.

During the 8th week of pregnancy, the volume of amniotic fluid is small (range 4-40 ml, see Chamberlain *et al.*, 1984; Brace and Wolf, 1989; Gilbert and Brace, 1993), and if leakage occurred at this time, it is unlikely that either the patient or her clinician would notice. Equally when vaginal bleeding occurs after *cvs*, any amniotic fluid loss is likely to remain hidden. In the MRC trial, vaginal bleeding was reported in 7% of women after transcervical *cvs* and in 2% after transabdominal *cvs* (MRC Working Party, 1991). While the overall incidence of amniotic fluid leakage reported in this trial was 0.43%, it is likely that this may occur considerably more often than these figures suggest, for the reasons indicated above. In an earlier study of 1000 consecutive cases of *cvs* (Hogge *et al.*, 1986), the overall incidence was 0.2%, while in 5 other cases evidence of severe oligohydramnios was noted when ultrasonography was carried out as a follow-up procedure at between 16-20 weeks of pregnancy. In the latter cases, "*there was no clinical history to suggest overt rupture of the membranes or leakage of amniotic fluid*."

It has recently been hypothesised that *cvs* may induce materno-fetal transfusion of serum which is immunologically active against fetal blood group antigens. In a clinical study in which two consecutive chorionic villus samples were taken from 18 women, a significant increase in maternal cells was observed in the fetal circulation in about 20% of cases following the second sample (Los *et al.*, 1996). It was therefore proposed that in some cases materno-fetal transfusion following *cvs* might induce maternal immunization against fetal antigens, and that this could provoke immunological damage to the fetus. In a model system designed to test this hypothesis, rat embryos with about 10 pairs of somites retained in whole embryo culture were given an intracardiac injection of antisera (van der Zee *et al.*, 1997). While the dorsal aorta at this stage of development has an intact endothelial lining, and the pharyngeal arch arteries and the limb vessels still have a fenestrated epithelium, it was proposed that this might lead to vascular disruption in the locality of the latter vessels, leading to the range of limb and craniofacial abnormalities seen following *cvs*. Since *cvs* is carried out clinically at much later stages of embryogenesis than studied in this model, it is unclear whether the immunological mechanism of action proposed by these individuals to account for the abnormalities observed following *cvs* is likely to be relevant clinically.

Recent recommendations regarding the optimal time during pregnancy for undertaking *cvs*, and its value in the early detection of genetic abnormalities

The findings from an extensive international clinical trial carried out between May 1992 and May 1994 under the auspices of the WHO, and involving 138,996 pregnancies in which *cvs* was undertaken primarily between weeks 9 and 12 of pregnancy, revealed no significant increase in limb defects above matched control levels (Froster and Jackson, 1996; Kuliev *et al.*, 1996). A total of 77 infants with limb defects were reported to the WHO CVS Registry, corresponding to an incidence of limb defects of between 5.2-5.7 per 10,000, compared with 4.8-5.97 per 10,000 in the general population. Analysis of the pattern distribution of the limb defects after *cvs* revealed no difference from the pattern in the general population. It was therefore strongly recommended that *cvs* should not be carried out before 9 weeks of pregnancy. Such a delay in undertaking this procedure also excluded cases of early fetal death which it was suggested could be diagnosed reliably by ultrasonography at 9 weeks of pregnancy. In Edinburgh, *cvs* is routinely undertaken at 11 weeks of pregnancy.

The timing and technique involved in undertaking this procedure is clearly of critical importance, principally because of the anatomy of the chorion in the first trimester of pregnancy. At about 5 weeks of pregnancy (3 weeks post-fertilization) the chorionic sac with its uniform covering of chorionic villi (the *chorion frondosum*) completely surrounds the embryo. Over the succeeding weeks, the villi facing the uterine cavity gradually degenerate, while those adjacent to the *decidua basalis* (or basal plate) proliferate to form the placenta. At about 8 weeks of pregnancy, the *chorion frondosum* at the placental site is about 3-6 mm thick, contains mitotically active villi and is the preferred biopsy site, while the *decidua capsularis* (at the site facing the uterine cavity, or *chorion laeve*), measures less than 2 mm in thickness. By 10 weeks, further thinning of the *chorion laeve* has taken place, and the villi associated with the *decidua capsularis* have almost completely disappeared (Lilford, 1990). It should not be altogether surprising therefore if occasionally the tip of the catheter inadvertently punctured the chorionic sac and entered the amniotic cavity. Indeed it is surprising that this does not happen more often.

With this in mind, the majority of the most recently published studies involving *cvs* have been undertaken after 9 weeks of pregnancy, and for the reasons indicated above are usually undertaken under ultrasound guidance. In a recently published textbook on prenatal diagnosis, while recommending that *cvs* should not be undertaken before 9 weeks of pregnancy, and preferably should be carried out at 10-11 weeks of pregnancy, provides neither anatomical nor other reasons why this procedure should not be undertaken earlier in pregnancy, only indicating that there is *no increased risk of limb defects following cvs* (Jackson and Wapner, 1993). These deficiencies are addressed in Wapner (1997), who is now of the view that the previously reported incidence of limb defects is probably accounted for by the fact that *cvs* was commonly performed too early in pregnancy in these earlier studies, and was carried out by individuals who were relatively inexperienced in the techniques involved. He states... "Sampling prior to 10 weeks' gestation should be limited to exceptional cases. *Cvs* beyond 10 weeks, in experienced hands, continues to have a low (if any) risk of fetal abnormalities and should continue to be routinely offered." The surface of the villi is covered by syncytiotrophoblast; its core contains mitotically active cells, and provides the tissue for direct preparation of karyotypes, whereas the mesenchymal core serves as a source of cells suitable for tissue culture and biochemical studies (Wapner, 1997).

Over the last few years, the availability of large numbers of sophisticated targeted gene probes has dramatically increased the

value of *cvs* as a diagnostic tool beyond its almost exclusive former use in the detection of cytogenetic anomalies during early pregnancy. A small selection of conditions where biopsies obtained via *cvs* have been analysed specifically to establish the presence of genetic mutational disorders follows, to give an indication of the scope and range of possibly more than a thousand direct mutation tests currently available worldwide: for the diagnosis of Marfan syndrome (Rantamaki *et al.*, 1995); glycogen storage disease type 1a (Qu *et al.*, 1996); Smith-Lemli-Opitz syndrome – 7-dehydrocholesterol reductase deficiency (Mills *et al.*, 1996); adrenoleukodystrophy (Imamura *et al.*, 1996; Rowland *et al.*, 1996); recessive dystrophic epidermolysis bullosa (McGrath *et al.*, 1996); beta-thalassaemia and sickle cell anaemia (Tuzmen *et al.*, 1996); hemophilia B (Young *et al.*, 1996); Crouzon disease (Schwartz *et al.*, 1996); triosephosphate isomerase deficiency (Arya *et al.*, 1996); X-linked hyper-IgM syndrome (Seyama *et al.*, 1996); osteogenesis imperfecta (Pepin *et al.*, 1997).

It should be emphasised, however, that even the majority of specialist centres are unlikely to have access to more than a limited number of these gene probes, and it might only be possible to carry out specific tests at regional and national centres. Scotland, for example, at the time of writing this review, has access to just over 50 direct mutation tests should they be requested. Some of the most commonly requested non-cytogenetic tests carried out on *cvs* samples in Edinburgh are for the following, with an indication (in parentheses) of how many of these tests were undertaken over the previous 10 months: routine testing for common cystic fibrosis mutations (17), alpha-1 anti-trypsin (5), Huntington's disease (4), myotonic dystrophy (2) and spinal muscular atrophy (1).

Justification for undertaking experimental animal studies

While the underlying mechanism of action of early *cvs* has yet to be fully determined, far fewer of these clinical investigative procedures are now undertaken *early* in pregnancy than formerly (see previous section), and the interest in determining the relationship between *cvs* carried out at between about 56-66 days of gestation and the birth of infants with OMFL syndrome-like defects appears to be diminishing, as the number of infants with *cvs*-induced craniofacial and/or limb abnormalities has correspondingly declined. While this may indeed be the case, it is still of critical importance to establish how *cvs*, which ostensibly involves the removal of a small sample of chorionic villus material, could interfere, albeit in a small proportion of cases, with the factors that control limb patterning, as well as inducing craniofacial defects.

Our interest in *cvs*-induced abnormalities was stimulated some years ago when browsing through the clinical literature on this topic, when it became abundantly clear that the majority of the clinical authors in this field appeared to be completely ignorant of the considerable volume of *extremely relevant* experimental animal literature involving ASP that, we believe, provides a means of investigating the underlying mechanisms involved in the induction of both the craniofacial and limb lesions observed following *cvs* (Kaufman, 1994). Had there been any awareness of the findings from these experimental studies undertaken during the previous 30 or more years, then it is likely, in our view, that the small but nevertheless *finite* risk of inducing these lesions when undertaking *cvs* during *early* pregnancy should have been appreciated by those organising these clinical trials. Equally, we were unhappy with the proposed mechanisms of induction of the defects (see, for example, Report of NICHHD Workshop, 1993, and observations relating to this publication noted above), as these were largely based on the findings obtained from a restricted number of rather extreme experimental animal studies that, in our view, were likely to be of only limited relevance.

We have proposed the hypothesis, as had previously been put forward by Shepard *et al.* (1991), that in a small proportion of cases in which *cvs* is undertaken, inadvertent puncture of the amniotic sac occurs, leading to an uncontrolled loss of amniotic fluid, and oligohydramnios in some cases. This hypothesis is based on the clinical information alluded to in the previous section regarding the difficulty often encountered in recognising amniotic fluid loss if it occurs following this procedure, particularly if it is associated with vaginal bleeding. It is also based on the type of palatal and limb abnormalities encountered in infants whose mothers were subjected to *cvs* during early pregnancy, as these closely resemble the type of abnormalities induced in experimental studies in which rodents have been exposed to ASP during a comparable stage of pregnancy.

Over the last few years, the genes involved in distal limb modelling have been investigated using animal systems (see below), and an analysis of their modified distribution following ASP is likely to shed important new light not only on the underlying mechanisms involved in inducing distal limb, including digital, defects following *ASP*, but also on the mechanisms underlying the similar range of distal limb defects encountered following *cvs*.

The experimental design

For reasons that are not entirely clear, very few publications using the technique of ASP have appeared since the mid-1980s, and with few exceptions in the previous literature, the rat has been used as the experimental animal of choice. Because we were more familiar with the early development of the mouse (see Kaufman, 1992), once it was decided to investigate this problem, it was first necessary to establish a mouse model in which a high incidence of both craniofacial and limb abnormalities could be induced. After undertaking a preliminary literature search, it became clear that when ASP was undertaken during Theiler Stages 20-22 (mouse: Theiler, 1989), approximately equivalent to Carnegie Stages 18-21 (human: O'Rahilly and Müller, 1987), lesions of the desired type might reasonably be expected to occur. It was then necessary to undertake this procedure on days 12,13,14,15, and 16 of pregnancy in the mouse to determine the post-operative survival rate, as well as the incidence and types of malformations induced. The day of finding a vaginal plug was designated as day 1 of pregnancy –this is also designated E 0.5 by some researchers (Kaufman, 1992). In all cases, ASP was undertaken on all of the conceptuses located in one uterine horn, while the embryos in the other uterine horn acted as 'internal' controls. The total number of resorptions and their location in the experimental horn was also noted. The 'internal' control group of embryos were from females exposed to a general anaesthetic and a laparotomy, but the uterine horns in which *they* were located were not exteriorised. In additional controls, females were exposed to a general anaesthetic, but not subjected to a laparotomy (termed the 'anaesthetic-only' control group). Because we were familiar with the use of Avertin (tribro-

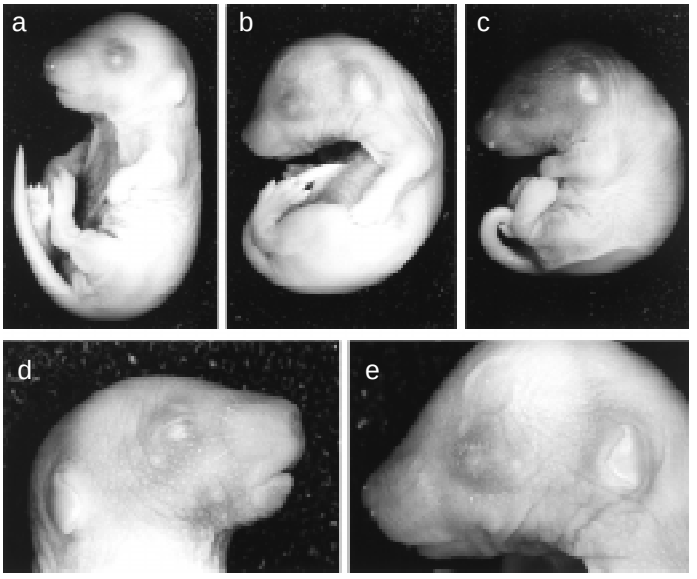

Fig. 1. Lateral views of an 'internal' control embryo (a) and two experimental embryos (b,c) previously exposed to ASP during the morning on day 13 of pregnancy. *All three were isolated on day 19 of pregnancy. The two experimental embryos display moderate to severe degrees of compression, and the hind limbs in both display evidence of the clubfoot deformity. In one of the experimental embryos* **(c)**, *the distal part of its tail shows an abnormal ventral spiral. In* **(d)**, *a close-up view of the head region of another 'internal' control embryo is shown in order to contrast it with the close-up side view of the head region of embryo* **(b)**, *which is seen to display an abnormal degree of reduction of the lower jaw profile (hypomandibulosis).*

moethanol), this was used as the general anaesthetic of choice in the majority of our studies (for details, see MacIntyre *et al.*, 1995).

When the preliminary experimental animal study was undertaken, it rapidly became apparent that the external diameter of the puncturing needle was also an important factor; highest rates of palatal defects and limb abnormalities were induced following ASP carried out using a 21 gauge needle (external diameter 0.65 mm) on the morning of day 13 of pregnancy. When a 25 gauge needle was used (external diameter 0.40 mm), the incidence of abnormalities induced was extremely low. The catheter commonly employed clinically to undertake *cvs* has an outer diameter of 2.1 mm (for details of methodology, see Elias and Simpson, 1987), while a 19 to 21 gauge needle is recommended for amniocentesis (Beischer and Mackay, 1986). While the survival rate was somewhat higher when ASP was undertaken on day 14, rather than on day 13 of pregnancy, the overall incidence of abnormalities of the palate, limbs and tail was substantially lower. All succeeding studies were therefore undertaken using a 21 gauge needle on the morning of day 13 of pregnancy; only spontaneously cycling (C57BLxCBA) F1 hybrid females mated to males of the same genetic background were used.

No attempt was made to isolate a finite volume of amniotic fluid as a component of the puncture procedure, and care was taken to avoid the location of the placenta and major blood vessels associated with the yolk sac, and traumatising the embryo with the tip of the needle. When the tip of the needle was withdrawn, an uncontrolled amount of leakage of amniotic fluid occurred, but no attempt was made to compress the sac to expel all or most of the amniotic fluid present, as was undertaken in some of the earlier studies (e.g., Walker, 1959; Singh and Singh, 1973).

The findings from the preliminary study

When ASP was carried out on the morning of day 13 of pregnancy on 102 embryos, 53% survived to day 19 of pregnancy and their external morphological features were analysed in detail. Of the latter, 35% were found to have a cleft palate, and 61% had one or more morphologically abnormal limbs; in addition, 43% had an abnormal tail. When ASP was carried out on the morning of day 14 of pregnancy on 83 embryos, 81% survived to day 19 of pregnancy, when their external morphological features could be analysed in detail. In the survivors, 27% had a cleft palate, 39% had one or more morphologically abnormal limbs, and 19% had an abnormal tail. In the 'internal' controls, of 86 and 61 embryos respectively isolated from the day 13 and day 14 mice, the survival rates were 97 and 90%, respectively. No evidence of palate, limb or tail abnormalities was seen in the two 'internal' control series.

In addition to the specific lesions of the palate, limbs and tail, the overall appearance of many of the experimental embryos displayed evidence of a mild to moderate, or a moderate to severe degree of compression (Fig. 1, see also MacIntyre *et al.*, 1995, Figs. 1 and 2). In the most extreme cases of external compression, the lower jaw was compressed on the upper part of the thorax, and this was associated with a considerable degree of micrognathia/hypomandibulosis (Fig. 1b,c,e; see also MacIntyre *et al.*, 1995; Figs. 2a-d). Occasionally, limbs were fused to other parts of the trunk or to the tail (Fig. 2a; see also MacIntyre *et al.*, 1995, Figs. 1a and b, 3k and l). Of greatest interest, however, was the range of abnormalities of the limbs and tail observed in this study. Following the proposal of Froster and Baird (1992), we decided to adopt a classification of limb abnormalities based on the suggestions of the American Society of Surgeons of the Hand and of the International Society of Prosthetics and Orthotics (Swanson, 1976). The majority of the limb abnormalities involved the handplate/footplate, and ranged from, in the most extreme cases, distal limb amputations (Fig. 3d-e), adactyly (absence of digits), syndactyly (fusion of digits) through to brachysyndactyly (shortened and fused digits), synonychia (fused nails) and diastodactyly (lateral divergence of two adjoining digits) (Fig. 3e-j), with the occasional fusion of individual digits or the whole handplate/footplate to other parts of the body (see above). Degrees of clubfoot/clubhand were also noted (Fig. 3a-g), but were not quantified in this study (see, however, next section). The tail abnormalities were also of interest and quite variable in their appearance, ranging from tails with a distinct single or double 'kink', to those in which the distal half was in the form of a complete spiral (see MacIntyre *et al.*, 1995; Fig. 4a-d).

In the case of the limbs that displayed *adactyly*, with the apparent absence of individual digits, subsequent studies on 'cleared' specimens stained to demonstrate the presence of skeletal elements (see below) revealed that in most cases *all* of the skeletal elements of the autopod were present. The adactyly commonly observed following ASP therefore appears almost exclusively to involve the soft tissues of the interdigital spaces; these fail to regress, as usually occurs during digit formation. These lesions need to be distinguished from distal limb *amputations*, which are far less commonly encountered in these studies.

In the day 13 series, a significant reduction in both the body weight and crown-rump length in those experimental embryos that displayed abnormalities of at least one system compared to the control series was also noted; such a difference in weight, but not in the crown-rump length, between these experimental and control embryos had previously been noted by Singh *et al.* (1974). When no obvious external abnormalities were present, their weight was not significantly different from control values, though the crown-rump lengths of both the 'normal' and 'abnormal' groups were significantly smaller than the matched controls.

Analysis of the postcranial skeletal elements in the experimental compared to the control embryos

A subsequent study was undertaken in order to examine the postcranial skeleton, but particularly the skeletal elements in the limbs, in those experimental embryos that displayed limb abnormalities. It was of interest to study those limbs with soft tissue lesions such as adactyly and syndactyly, in order to establish whether these lesions were associated with underlying skeletal lesions, such as deficiencies of the phalanges or metacarpals or metatarsals. To our surprise, in the majority of cases, all of the skeletal elements were present. In only a relatively small proportion of cases were the distal phalanges abnormally shaped, and in some cases either directly fused together, or joined via interdigital skeletal 'bridges' (Chang *et al.*, 1996). In the initial study undertaken to establish the methodology, it was apparent that a proportion of the embryos with abnormal limbs displayed the clubfoot deformity. Only when these embryos were cleared and double-stained to demonstrate the skeletal elements was it possible to quantify the true incidence of this condition. Moreover, it also became apparent that a similar deformity of the wrist region was also revealed, a condition termed clubhand (syn.: clubwrist).

When these deformities of the ankle and wrist regions were carefully analysed and scored according to their degree of severity by two independent observers, a high degree of concordance was obtained. This analysis also demonstrated that for clubhand, the left forelimb was often more severely affected than the right forelimb; the incidence of clubfoot was similar between the two sides. In some of the examples of clubfoot, a deformity of the shape of the calcaneus, which had an exaggerated arch-like shape, was also observed. In some cases of clubfoot seen clinically, an abnormality of the shape of the talus/calcaneus is observed which is believed to be genetically determined, and these are found to be particularly resistant to correction even by operation (Palmer, 1964; Wynne-Davies, 1964a,b).

The availability of the cleared and double-stained specimens also allowed measurements to be made of the individual bones of the forelimbs and hindlimbs. The reduction in the length of the longbones observed in the experimental series was found to be consistent with an overall degree of growth retardation in these embryos (Patton and Kaufman, 1995; Chang *et al.*, 1996), and was consistent with the significantly reduced crown-rump length and body weight of the 'abnormal' experimental compared to the control embryos.

The most unexpected skeletal finding, however, was the association between gross morphological abnormalities of the palate, limbs and tail, and abnormalities of ossification of the sternum. In only 13 out of 38 embryos examined in the experimental series, the appearance of the ossification centres appeared to be similar to the normal pattern. Thus, in those experimental embryos in which there were no external morphological abnormalities seen, the appearance of their sternal ossification centres was similar to the normal pattern. In the remaining 25 cases, the pattern of ossification seen was in most instances extremely bizarre, often involving the fusion between two or more adjacent sternebral ossification centres. Occasionally, all of the sternebral ossification centres were fused together (a selection of the anomalous sternal ossification patterns seen is presented in Chang *et al.*, 1996, Fig. 3).

It has long been known that the sternum and ribs are particularly prone to disruption during development (Grüneberg, 1963; Johnson, 1986). Grüneberg was certainly of the opinion that the sternal abnormalities seen were usually secondary to rib anomalies in which the ribs failed to make contact with the sternal bands as normally occurs (Green and Green, 1942). In this instance, it is possible that the change in amniotic fluid pressure following ASP may interfere, either directly or indirectly, with the normal expression pattern of certain critical pattern-forming genes such as *Engrailed-1* (Wurst *et al.*, 1994) that control the ossification of the sternal elements, possibly in an analogous way to the situation observed with regard to the interdigital zones.

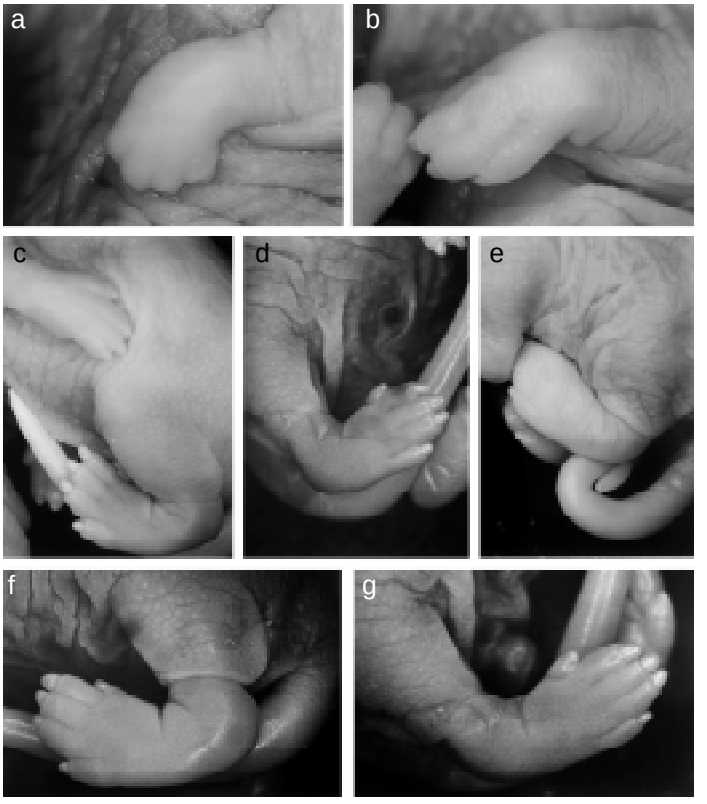

Fig. 2. Two views of forelimbs (a,b), and five views of hindlimbs (c-g) of experimental embryos isolated on day 19 of pregnancy to display the typical postural defects observed in such embryos. *The two forelimbs* **(a,b)** *both display evidence of a clubfoot/clubwrist deformity, while the hindlimbs shown here display the typical features of the clubfoot deformity commonly seen in the experimental embryos following ASP. The hindlimbs illustrated in* **(c)** *and* **(e)** *are from the intact mice shown in Figures 1 b and 1c.*

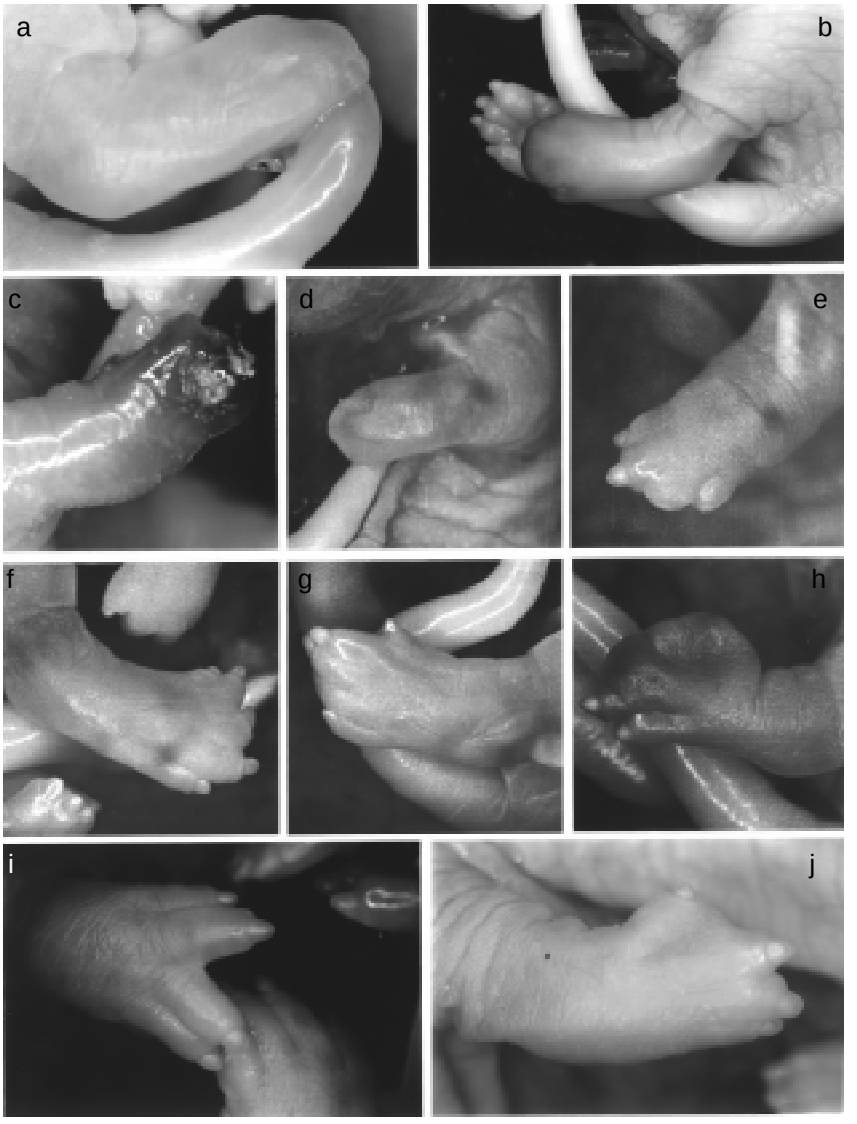

Fig. 3. Representative selection of limb abnormalities seen in experimental embryos isolated on day 19 of pregnancy. *Fusion between a hindlimb and the tail* **(a)**, *and three examples of amputations, two of the hindlimb* **(b,c)**, *and one example of a forelimb amputation* **(d)**. *In the example illustrated in* **(c)**, *the terminal part was haemorrhagic and appeared to be covered by necrotic tissue. In the latter 3 examples, the impression was gained that the distal skeletal elements were missing. The other examples* **(e-j)** *illustrate the typical type of digital abnormalities encountered, most of which display some evidence of syndactyly. The limb illustrated in* **(h)**, *shows an extensive area of bruising with disruption of the normal digital anatomy, while the limb illustrated in* **(i)** *shows lateral divergence of two adjacent digits (diastodactyly). The abnormalities displayed here complement those illustrated previously (see MacIntyre et al., 1995, Fig. 3).*

In the mouse, the ribs normally make contact with the sternum at about day 13, while the "zipping up" of the ladderlike sternal rudiments is initiated during day 14 (Chen, 1952). The timing of these critical events would therefore make them particularly sensitive to adverse environmental factors acting at this time. The asymmetrical distortion of both the rib cage and sternebral elements observed in our study could easily have a postural basis, resulting from pressure secondary to amniotic fluid loss (Chang *et al.*, 1996), though clearly a genetic basis for the abnormal pattern observed, through interference with the genes involved in these processes, cannot yet be excluded.

In some mice with syndactylism (*sm/sm*, Grüneberg, 1956), there is an associated involvement of the tail; in these mice, fusion between adjacent phalanges is observed, whereas the metacarpals and metatarsals remain separate. The apical ectodermal ridge in these mutants is noted to be thicker than in their normal littermates (Grüneberg, 1960). The syndactyly observed following ASP appears to exclusively involve the mesenchyme tissue of the interdigital zones, though the epidermis in these regions may be hypertrophied (authors, unpublished observations). Usually there is no effect on the phalanges, although occasionally the distal phalanges may be morphologically abnormal or fused to each other by inter-phalangeal 'bridges.'

It is possible that the tail abnormalities observed may also result from interference with the normal functioning of pattern forming genes associated with the axial skeleton; in this instance, inducing either extensive unbalanced shortening of the ventral caudal tendons (in those with a complete spiral) or localised regions of shortening of the lateral caudal tendons (in those with a single or double 'kink'). The total number of caudal vertebrae present may be normal, as occurs in *screw-tail* mice (MacDowell *et al.*, 1942), or grossly abnormal and/or reduced in number, as occurs in the *tail-short* mutant (Deol, 1961), and as seen in some of our abnormal mice.

Evidence of a transient period of bradycardia following ASP

Because we had previously noted that when embryos are analysed shortly after ASP, they commonly displayed extensive areas of cutaneous bruising or haemorrhages (ecchymoses) involving the head region, trunk and limbs, this led us to suspect that this might be secondary to vascular stasis, with pooling of blood in the venous system and capillaries (Chang and Kaufman, 1997); similar extensive areas of ecchymoses had also been noted by others in their experimental studies shortly after ASP (e.g., Singh and Singh, 1973; Kennedy and Persaud, 1977; Houben, 1984), and on one occasion clinically shortly after *cvs* when a fetus was viewed by embryoscopy (Quintero *et al.*, 1992). It is of interest that a mouse mutant has recently been described (Seller and Wallace, 1993) that characteristically displays haemorrhagic lesions and subsequently transverse reduction deformities of the tail, and this has been proposed as a possible model for a variety of syndromes which display transverse reduction defects of the limb.

While others had speculated that vascular disruption might have occurred in response to the removal of a small chorionic villus sample, causing either fetal hypotension, or possibly hypoperfusion and anoxia due to fetal blood loss, or thrombus formation at the site of biopsy with distal embolization (Firth *et al.*, 1991b; Burton *et al.*,

1992; Brent, 1993; Holmes, 1993; Report of the NICHHD workshop, 1993), we were not convinced that localised sites of tissue ischaemia that were believed to develop in the embryo secondary to placental trauma (due to the blocking of peripheral blood vessels by displaced thrombus material from the placenta) provided an adequate explanation for the clinical findings reported following *cvs*. We hypothesised that the ecchymoses consistently observed shortly after ASP might have been due to venous stasis, and resulted in hypoperfusion of tissues, and that this might have resulted from a transient episode of bradycardia.

The fact that no *direct* evidence of a relationship between a transient period of fetal bradycardia and *cvs* had been reported, could in our view have had a number of explanations. For example, the fact that a relatively low incidence of fetal abnormalities has been reported following *cvs* might be indicative of the fact that in most cases when fetal monitoring was performed *cvs* had been undertaken after day 66 of gestation (Kofinas *et al.*, 1995). It is also relevant to note that this phenomenon might not have been seen due to the small sample sizes in most studies (Zoppini *et al.*, 1993; Hibbard *et al.*, 1994; Brezinka *et al.*, 1995). Another possible problem might arise because of the *delayed* onset of bradycardia which would not be noted if monitoring of the fetal heart rate were made only in the *immediate* post-*cvs* period (Burton *et al.*, 1992).

As an example of studies with a small sample size, Hibbard *et al.* (1994), using Doppler velocimetry, investigated fetal heart rate directly after *cvs* on 21 consecutive individuals, but in all cases failed to discover any significant difference in values before and immediately after *cvs* when the data were analysed by gestational age, villus biopsy sample size, method (i.e., transabdominal or transvaginal *cvs*), placental location, or operator. Similarly, Brezinka *et al.* (1995) studied the Doppler flow waveform in the fetal ductus venosus and umbilical artery in 36 women of advanced maternal age who were subjected to transabdominal *cvs* at between 11 and 13 weeks of gestation. Bradycardia and an abnormal cardiac response was, however, noted in one case, and resulted in fetal loss one week later; the appearance of the limbs was not noted. Kofinas *et al.* (1995) examined 42 patients in which *cvs* was undertaken between 9.5 and 12 weeks of gestation, and found no evidence of bradycardia following *cvs*. In all of these studies, however, *cvs* and cardiac monitoring was undertaken well beyond the day 56-66 'window' when limb abnormalities are most likely to occur.

Despite the fact that this phenomenon had not previously been noted when embryonic/fetal heart rates were monitored immediately after *cvs* (see above), we decided that it would be appropriate to measure *directly* the heart rate of control and experimental embryos isolated at specific times after ASP using time-lapse cinephotomicrography. Four groups were studied: (i) embryos isolated from females at about 10.00 on the morning of days 12-15 of pregnancy that had not been exposed to any experimental procedure, this constituted the non-experimental control group, (ii) embryos isolated from females during the morning of day 13 of pregnancy at 30 min, 2h and 4h following the anaesthetic injection, and this constituted the anaesthetics-only control group, while females in groups (iii) and (iv) were exposed to a laparotomy, and constituted the experimental groups. Those in group (iii) were exposed to ASP, while embryos in the contralateral uterine horn [group (iv)] were left undisturbed, the embryos acting as 'internal' controls.

Uterine horns were opened carefully and rapidly, and decidual swellings isolated as an intact unit without damaging either the placentas, the embryos or their extraembryonic membranes. These units were immersed in normal saline buffered with 25% fetal calf serum, and maintained at 37°C on a heated stage in a heated cabinet and viewed by time-lapse cinephotomicrography for 75-90 sec. By this means, the heart rate (beats/min) could be accurately determined. In embryos from group (i), the heart rate could be determined by analysing the pulsation of the umbilical cord or by the movement of the whole body (Suzue, 1994). Following ASP, it was necessary to expose the embryo in order to view the pulsation of the heart chambers more precisely, care being taken when doing so to maintain an intact circulation. Only embryos with sinus rhythm were considered in the main study, though the incidence of dead embryos and those with cardiac arrhythmias or evidence of a heart 'block' was also determined. The incidence and an indication of the extent of ecchymoses present was also determined in each of the groups studied.

This study demonstrated that there was an *increased duration* of bradycardia observed in the experimental group exposed to ASP, *beyond that observed in those embryos exposed to anaesthesia alone*, in the anaesthesia-only controls, and in the embryos isolated from the contralateral (non-operated) uterine horns. We believe that this effect on heart rate is likely to be secondary to compression of the embryo by the extraembryonic membranes and uterine muscle following ASP, being a consequence of oligohydramnios. In the light of this finding, we have proposed the hypothesis (Chang and Kaufman, 1997) that hypoperfusion of the peripheral part of the developing limb bud either (a) interferes with the normal pattern of apoptosis that occurs in the digital interzones, or (b) induces an abnormal degree of cellular proliferation and/or tissue regeneration in these sites, possibly because of the overexpression of certain genes, or due to the presence of growth factors brought to the vicinity by macrophages during their clearing of the necrotic tissue (Hopkinson-Woolley *et al.*, 1994).

Possible effects of cutaneous haemorrhages commonly seen following ASP and *cvs*

Cutaneous haemorrhaging has been observed in human limbs following *cvs* (Quintero *et al.*, 1992; for details, see previously), and in rat embryos after uterine clamping (Webster *et al.*, 1987) or amniotic puncture (Kennedy and Persaud, 1977; Houben, 1984). Such cutaneous lesions are also commonly seen in our studies following ASP (see previously). Release of blood in ecchymotic lesions could induce macrophage recruitment to the site. Macrophages are also found in the interdigital regions as apoptosis is initiated. Although no published information is available for embryonic macrophages, in the adult, macrophages provide a source of growth factors and cytokines, and cells at the edges of embryonic wounds, in the absence of macrophages, are known to upregulate at least one growth factor, TGFβ 1 (Martin *et al.*, 1993). The up- or down-regulation of genes regulating apoptosis may be affected, for example, TGFβ1-soaked bead implants inhibited proliferation and apoptosis and induced ectopic cartilages in the chicken hindlimb (Gañan *et al.*, 1996). Up-regulation of FGFs, which can act antagonistically to BMP proteins (also members of the *TGFβ* gene family), may also be involved. It is possible that such a mechanism may be involved in the inter-digital abnormalities observed following ASP.

Histological analysis of the interdigital zones in control and experimental limbs isolated at various times after ASP

It is important to note that, apart from the abnormalities of the ankle and wrist regions, the clubfoot and clubhand deformities, which are probably of postural origin, the majority of the limb abnormalities observed involved the soft tissues of the interdigital zones. The clearing and double-staining studies (see previously) revealed that, apart from the occasional incidence of fusion of two or three of the distal phalanges, syndactyly usually only affected the soft tissues within the interdigital spaces. This was also observed in cases of adactyly: while the skeletal elements of the digits were invariably present, the soft tissues in the interdigital spaces failed to separate. As the most likely aetiology in these cases is interference with the normal pattern of programmed cell death (i.e., apoptosis), or possibly an increased degree of cellular proliferation, in the interdigital zones, an attempt was made to analyse these regions histologically at specific time intervals after ASP, with the aim of quantifying the cellular activity in these locations at these various times, and comparing the findings with those from comparable regions of matched control material. A preliminary study was undertaken using Bouin's fixed material, and in a subsequent study, semi-thin plastic sections were analysed.

No difficulties were encountered in determining the incidence of mitotic activity in the most peripheral part of the limb bud in the proximity of the marginal vein, and in the rest of the interdigital zones, because of the ease with which mitotic figures could be recognised. Because only conventional histological techniques were used, difficulties were encountered in *quantifying* the number of apoptotic cells present, and any assessment made could therefore only be qualitative in nature. Should this component of the study be repeated, it is recommended that specific stains should be used to unequivocally demonstrate the presence of apoptotic cells (see Coucouvanis *et al.*, 1995; Kerr *et al.*, 1995). While the aetiology of syndactyly appears to be reasonably straightforward, that in the case of diastodactyly is less clear, and may result from either the precocious loss of interdigital tissue due to increased cell death, or another more complex mechanism.

Histological analysis of the interdigital spaces in limbs isolated at 0.5, 4, 8, 12, 24 and 36 h following ASP revealed the occasional incidence of vascular disruption in the form of localised areas of haemorrhage, vascular dilatation and congestion and the presence of fluid-filled cavities in close proximity to the marginal vein and the vascular plexuses in the interdigital spaces. More commonly, apoptosis observed within the mesenchyme cells of the interdigital spaces was already inhibited in the majority of the experimental limbs analysed 4 h after ASP. Instead of undergoing necrosis/apoptosis, *increased* mitotic activity was usually observed from 8 h following ASP at sites where apoptosis would normally be expected to be seen. Vascular congestion and/or dilatation appeared to influence mitotic activity in the proximity of the marginal vein and its associated vascular network (Chang *et al.*, 1998). Semi-thin sections of additional material fixed for transmission electron microscopy and embedded in plastic, confirmed the findings from the conventional histological analysis, and also revealed that in the interdigital zones of the experimental but not in the control embryos there was considerable evidence of epithelial hypertrophy (authors, unpublished observations).

Inhibition of apoptosis in the interdigital zone has been observed following the removal of the apical ectodermal ridge in the chick (Hurle and Gañan, 1987) or after the injection of Janus Green B into the amniotic cavity (Fallon, 1972; Fernandez-Teran and Hurle, 1984). In both cases, the apoptotic programme in the interdigital zones was inhibited. Mesenchymal cells in these regions, instead of dying, differentiated to form ectopic cartilaginous elements, similar to the skeletal 'bridges' between the distal phalanges observed in our earlier study (Chang *et al.*, 1996).

Since it is of importance to establish whether the structural digital abnormalities observed are due to alterations in the apoptotic programme rather than to necrosis, histological sections should be stained with either the fluorescent DNA-binding dye propidium iodide (or DAPI), or haematoxylin and eosin/toluidine blue, both of which allow condensing chromatin in apoptotic versus necrotic cells to be differentiated at the light microscopic level (Coucouvanis *et al.*, 1995; Kerr *et al.*, 1995).

Analysis of whole -mounts of control and experimental limb buds using Nile blue sulphate

Preliminary studies were undertaken in which whole mounts of intact control and experimental limb buds were stained with Nile blue sulphate at specific time points after ASP. While this staining technique does not distinguish between necrotic and apoptotic cells, it has formerly been used to study cell death in the interdigital zones (Saunders, 1966; Saunders and Fallon, 1966). It still remains unclear exactly how the stain is taken up by dead/dying cells. It has, for example, been suggested that the stain is excluded by the intact cytoplasmic membrane of living cells, while in dead/dying cells the stain is able to permeate through the outer membrane of the cell. Apoptotic cells are engulfed by macrophages which are then identified by the affinity of the ingested débris for Nile blue sulphate (Saunders, 1966; Hopkinson-Woolley *et al.*, 1994).

In this investigation, limb buds were isolated at 24, 36 and 48 h after ASP, and a staining pattern consistent with developmental delay was particularly noted, with the proximal-distal length of the interdigital zones being substantially greater in the experimental than in the control embryos. In general, the average volume of cell death (as extrapolated from the area of Nile blue sulphate stained cells on photomicrographs of appropriately fixed and stained material) within the interdigital zones was lower in the experimental compared to control limbs, this feature being particularly evident in relation to the 2nd, 3rd and 4th spaces (Fig. 4a-f) (Verma, 1996).

Analysis of genetic activity in the interdigital zones

A reduction in the level of growth factors available and/or modifications in extracellular matrix molecules are thought to be involved in the normal activation of genes that control apoptosis in the limb (Hurle *et al.*, 1995). We have hypothesised that ASP either directly or indirectly disrupts the strict temporal and spatial regulation of apoptosis through a mechanism resulting from an alteration in the normal vascular supply to this region. Following specification of limb pattern and regression of the apical ectodermal ridge, interference with the expression of several patterning genes that are normally retained in the interdigital region may occur; the following are believed to be particularly important: *Msx1* and *2* (Robert *et al.*, 1989; Davidson *et al.*, 1991; Reginelli *et al.*, 1995),

Bmp2, *4* and *7* (Jones *et al.*, 1991; Francis-West *et al.*, 1995; Lyons *et al.*, 1995) and *Fgfr1* and *2* (Orr-Urtreger *et al.*, 1991; Peters *et al.*, 1992).

While we have only studied the expression pattern of the gene *Msx-1* in the interdigital zones of both control and experimental embryos, the most marked difference was the extreme degree of over-expression observed in the interdigital zones of the experimental compared to the control material (see Fig. 5a,b).

Effect of anaesthetic agent used during ASP on the survival rate of experimental embryos and the incidence and type of limb abnormalities observed

In all of our previous studies into the effect of ASP carried out on day 13 of pregnancy in mice, we have used intraperitoneal Avertin (tribromoethanol) as the general anaesthetic. In this study (Kaufman and Chang, 1998), we used an inhalational anaesthetic (a mixture of halothane, oxygen and nitrous oxide in a ratio of 2:3:3). The principal difference between these two regimens is that even under optimal post-operative conditions when Avertin is used it can take between 30-90 min before complete recovery is achieved; when the inhalational anaesthetic is used, complete recovery is usually achieved within about 3-5 min. Because the experimental conditions were otherwise identical, this allowed the influence of the anaesthetic employed on survival rate to day 19 of pregnancy, the incidence of cleft palate and the incidence and types of limb abnormalities observed, mean weight and crown-rump length of experimental compared to 'internal' control embryos (i.e., from the contra-lateral, non-operated, uterine horn), to be established.

The survival rate to day 19 of pregnancy was slightly higher when the inhalational anaesthetic was used, as was the incidence of limb abnormalities observed. The *overall incidence* of gross abnormalities was not significantly different, although the incidence of the various types of defects encountered did vary between this and the earlier Avertin study. The most marked difference, however, was in the incidence of syndactyly, which was significantly lower when the inhalational anaesthetic was used: 26.6% v. 70.2% of the abnormal limbs analysed. Since the only difference between these two studies relates to the type of anaesthesia employed, with the much shorter duration of the recovery period observed when an inhalational anaesthetic was used, rather than intraperitoneal Avertin, we believe that this provides us with a likely explanation for the difference observed in the incidence of syndactyly in these two studies.

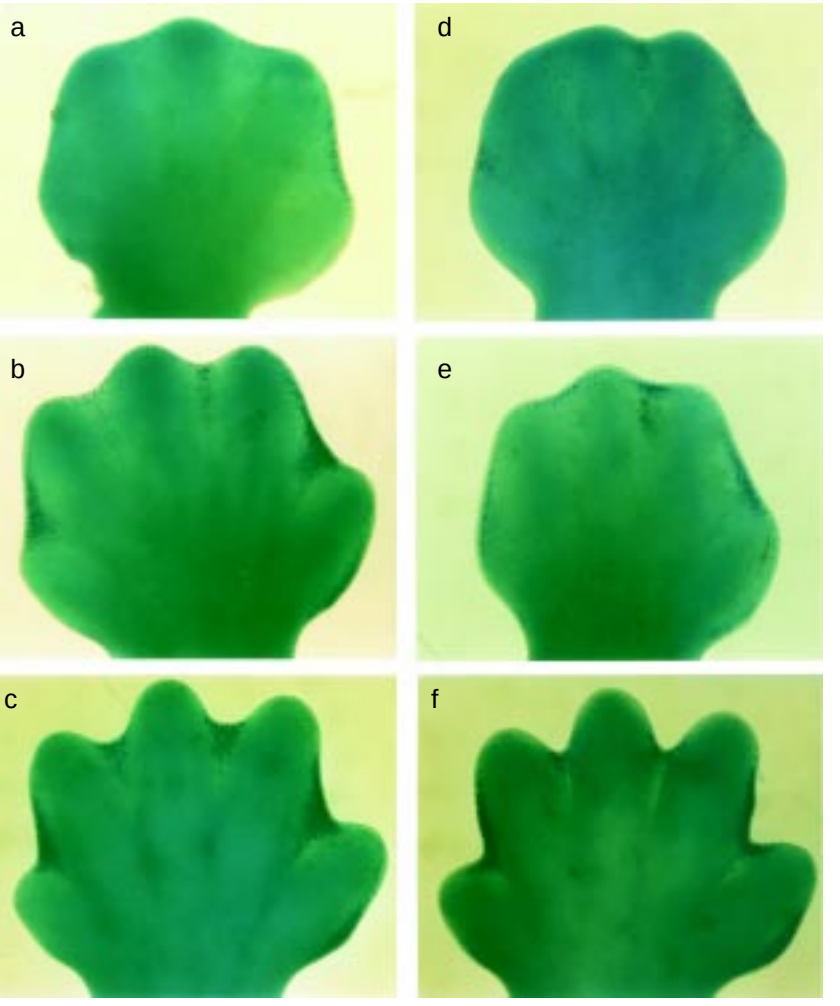

Fig. 4. Representative selection of dorsal views of whole-mounts of right hindlimbs from an experimental series (anaesthetised with Avertin) isolated at 24 h, 36 h and 48 h after ASP (d-f), with matched 'internal' controls (a-c) all stained with Nile blue sulphate to display the location of necrotic and apoptotic cells. *Note that the stained cells are principally located in the interdigital zones, and are also seen in the lateral margin of the autopod. In the 24 h control limb* **(a)**, *the Nile blue sulphate stained cells are principally located at the peripheral margin of the interdigital zones. In the matched experimental limb* **(b)**, *there appears to be an increased number of stained cells in these regions. This is likely to be due to the presence of large numbers of necrotic rather than apoptotic cells. The shape of the peripheral margin in the region of the second digit in* **(b)** *is abnormal, however, and suggests that syndactyly might have been observed between the first and second, and between the 2nd and 3rd digits had this embryo survived to day 19 of pregnancy. By 36 h after ASP, the area occupied by stained cells in the interdigital zones is considerably greater in the control* **(b)** *than in the experimental limb* **(e)**. *At 48 h, the difference is even more marked, so that by this stage there is a 'significant' reduction in the area occupied by stained cells in the experimental* **(f)** *compared to the control limb* **(c)**. *Had more advanced stages been analysed by this technique, the proximal-distal depth of all of the interdigital zones would have been significantly greater in the controls than in the matched experimental autopods.*

Based on the information obtained from our previous studies, we would wish to tentatively suggest that the explanation for the significant reduction in the incidence of syndactyly observed in the present compared to the previous study is likely to be related to the much briefer duration of the post-operative period when the inhalational anaesthetic agent was employed. We have previously noted (see above) that following an intraperitoneal injection of Avertin, not only is the duration of the post-operative period far longer, but this is associated with a considerably delayed period of bradycardia beyond that observed when embryos are exposed to Avertin alone (Chang and Kaufman, 1997).

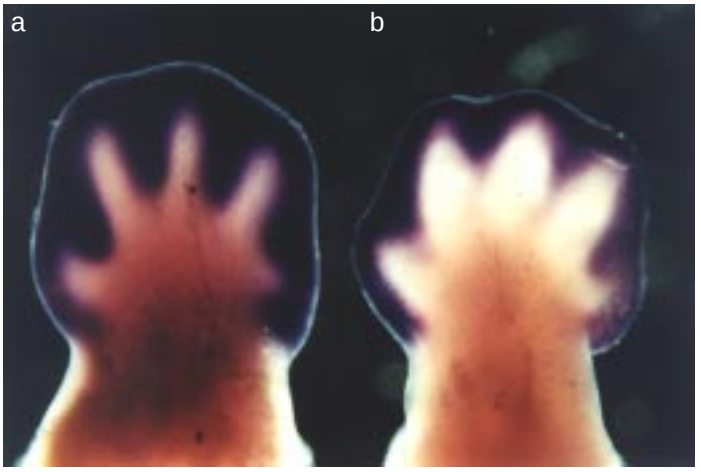

Fig. 5. A hindlimb isolated from an experimental embryo analysed at 4 h after ASP (a) to illustrate the expression pattern of the gene *Msx-1,* with a matched hindlimb isolated from an 'internal' control embryo (b) isolated from the contra-lateral uterine horn of the same female. *Note the extreme degree of over-expression of the gene in the interdigital zones and at the peripheral margin of the footplate in the experimental compared to the control limb.*

The substantially lower incidence of tail abnormalities observed in the present compared to the previous study, we believe, may also be explained by the improved vascular perfusion of the tail, and is consistent with our hypothesis relating to the lower incidence of syndactyly which was also seen in the present compared to the previous study. Another as yet unexplained finding in this compared to the Avertin series was the apparent increased degree of compression observed in a high proportion of the embryos that demonstrated abnormalities of one or more system. In many of these embryos, the palatal defect was associated with an extreme degree of reduction of the lower jaw profile, termed hypomandibulosis.

We believe that the findings presented here provide confirmatory, albeit indirect, information on the possible mechanism of induction of syndactyly in a high proportion of those cases where ASP was undertaken under Avertin anaesthesia where the post-operative period is of considerable duration. While we have yet to undertake studies in which the heart rate of embryos is analysed at specific time intervals following this procedure, as we have previously undertaken following Avertin anaesthesia (Chang and Kaufman, 1997), we believe that this will demonstrate that the duration of bradycardia is likely to be of considerably shorter duration following inhalational anaesthesia.

Conclusions

While it may never be possible to prove or disprove our hypothesis that the craniofacial and limb abnormalities occasionally observed following *cvs* undertaken during *early* human pregnancy results from the inadvertent puncturing of the amniotic sac in these cases, we believe that all of our experimental studies indicate that this is a possibility worthy of serious consideration.

We believe that the findings from our various studies have helped to shed new light on the mechanism of induction of syndactyly and related digital defects both clinically and in mammals. While previously it had not been possible to induce syndactyly experimentally with a high degree of efficiency except in avian embryos (Fallon, 1972; Fernandez-Teran and Hurle, 1984; Hurle and Gañan, 1987), we have recently demonstrated that if ASP is carried out on the morning of day 13 of pregnancy in mice using Avertin anaesthesia, a high proportion of the digital limb defects encountered are of this type. Although the exact mode of action still remains unclear, it appears likely that the ASP-induced bradycardia following Avertin anaesthesia causes vascular dilatation and congestion, as well as localised areas of haemorrhage in the proximity of the marginal vein and the vascular plexuses in the interdigital spaces. The resultant hypoperfusion and diminished oxygen supply to the interdigital tissues almost certainly interferes, either directly or indirectly, with the normal temporal and spatial regulation of the genes that control apoptosis in the interdigital zones.

The present findings are clearly of interest in explaining the underlying mechanism of induction of syndactyly following ASP carried out under certain experimental conditions. They also raise interesting points of principle particularly with regard to the interpretation of other experimental studies carried out under general anaesthesia where a prolonged post-operative recovery period may have secondary effects on the embryonic heart rate, and consequently influence the outcome of the study. This is particularly likely to be the case in teratological studies carried out under general anaesthesia on pregnant animals involving the period prior to and during organogenesis.

Acknowledgements

We thank Mr. Amit S. Verma for observations relating to the staining of the limb buds with Nile blue sulphate, and Ms. Julie Gordon for undertaking the semi-thin histological sections of the interdigital zones. These studies were undertaken as part of their BSc Honours Anatomy projects carried out under our supervision. We are also particularly grateful to Dr. Clare Gribbin for her invaluable advice and critical observations on the manuscript, and Mr. Nigel Bain for care taken in printing the photographs.

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