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In Vitro Culture of Neurons from Sheep with Batten Disease

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Specific storage of mitochondrial ATP synthase subunit c occurs in most forms of Batten disease, including the ovine form, but its relationship to the characteristic neurodegeneration is not clear. Storage occurs in most cell types but only neurons are functionally affected. Neurons were cultured from control and affected sheep. Ewes were superovulated and inseminated, and embryos were collected, frozen, stored, and later transplanted into surrogate dams for gestation at times to suit experimental demands. The optimal fetal age for cultures was investigated, from 50 to 125 days. There were no differences between control and affected embryos in this period of rapid growth. At 50 days brains consist of smooth-surfaced hemispheres and cerebellum with no obvious demarcation between gray and white matter. At 90 days they are like miniature adult brains. From 200 to 600 million viable cells were recovered from each fetus, regardless of age. DMEM/F12 with B27 was the most practical medium tested. Cell viability was not as good in medium containing serum. Treatment of surfaces with polylysine aided neuron adhesion. No developmental or viability differences were observed between normal and affected neuron cultures. At plating out cells were rounded. A day later single process outgrowths began. After 4 days these were over 200 μm and by Day 6 had created a network. Most neurons were bipolar. Neurons from 50 to 90-day old fetuses persisted in culture for over 100 days.

Key Words: **neuron culture; sheep fetuses; Batten disease; neuronal ceroid lipofuscinosis.**

Batten disease (neuronal ceroid lipofuscinoses, NCLs) is a group of inherited neurodegenerative lysosomal storage diseases. Characteristic pathological features are brain atrophy, consequent to neurodegeneration, and accumulation of fluorescent storage bodies in neurons and most other cells. Batten disease also occurs in animals, including a form in sheep which have been bred and studied as a model of the human diseases (1–3). Mutations in different genes underlie different forms of the disease, the gene in affected sheep being syntenic to that for the human CLN6 variant late-infantile form (4).

More than two-thirds of affected sheep storage bodies is protein, mainly subunit c of mitochondrial ATP synthase (5–7). This protein is also specifically stored in most of the human forms (8,9), suggesting that underlying them are lesions in the turnover of subunit c such that it accumulates in lysosome-derived organelles. This pathway is specific for subunit c, and subunit c accumulation is specific to NCLs (10,11).

Neurons cultured from the sheep model offer a good system in which to study several major unresolved issues. The relationship between protein storage and the mechanism of neurodegeneration can be studied in them. They live long enough for subunit c turnover studies. Extended cultures of sheep neurons have not been reported previously. In most species fetal neurons culture best. Sheep are naturally seasonal breeders so the first step was the development of a system that allowed fetuses to be harvested year round, at times to suit experimentation.

MATERIALS AND METHODS

Sheep. Affected, control, and heterozygous sheep are maintained on pasture at Lincoln University.

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Homozygously affected animals are produced by mating heterozygous ewes with affected rams to produce 50% diseased lambs, diagnosed by histopathology of brain biopsies (12).

Semen collection and semen freezing. Six-month-old homozygous affected rams were trained for semen collection using an artificial vagina, and semen was collected twice weekly. Ejaculates were diluted with egg yolk extender and frozen in 0.25-ml straws (13).

Superovulation, embryo collection, and embryo freezing. Affected and normal embryo donor ewes were synchronized with progesterone-impregnated controlled intrauterine drug-release devices (CIDRs) for 13 days. From Day 10 ewes received twice daily injections of 1 mg of follicle stimulating hormone (FSH, Ovagen, ICP, Auckland, NZ). After 4 days of FSH treatment, and 36 h after CIDR removal, laparoscopic intrauterine insemination was performed under local anesthetic. Embryos were recovered 6 days later by retrograde flushing of uterine horns with 20 ml Dulbecco phosphate-buffered saline (DPBS) containing 0.4% bovine serum albumin, introduced near the utero-tubule junction under general anesthetic. Embryos of suitable quality in the morula to blastocyst stages were equilibrated in 1.5 M ethylene glycol, frozen in straws (14), and stored in liquid nitrogen.

Generation of fetuses. Normal and affected embryos were developed in normal surrogate ewes, synchronized as above, at any time of the year. Frozen embryos were thawed rapidly at 37°C, equilibrated in 0.25 M sucrose for 10 min and then in DPBS containing 20% fetal calf serum (FCS), and then cultured overnight in drops of M199 containing 10% FCS, under oil, at 37°C in 5% CO_2 and maximum humidity. Embryos that reexpanded overnight were transferred semisurgically into the uterine horn ipsilateral to the corpus luteum of the synchronized recipients. Pregnancy was confirmed by ultrasound scanning after 50 days gestation.

Fetal harvesting and brain cell preparation. Pregnant ewes were slaughtered by captive bolt and exsanguination, and the fetuses were removed and exsanguinated. The cranium was immediately opened, the brain transferred to sterile DPBS containing antibiotics at 4°C, the meninges removed, and the tissue chopped finely. Cold Krebs Ringer buffer was added (10 ml/g tissue) followed by trypsin (Sigma) to 0.025%, and the suspension was incubated overnight at 4°C. Digestion was carried out at 37°C for 30 min with shaking. DNase (Sigma) and soya bean trypsin inhibitor (Sigma) were added to 0.006 and 0.1%, respectively, and the suspension was incubated at 20°C for 10 min. After gentle trituration with a fire-polished Pasteur pipette aggregates were allowed to settle for 3 min and cells were harvested from the supernatant by centrifugation, resuspended in culture medium (10 ml/g starting tissue), and then kept in a CO_2 incubator until plating out. Live cells were recognized by trypan blue exclusion and counted.

Cell culture. Cells were cultured at a density of 50,000 cells/cm^2 on glass microscope slides and coverslips submerged in Petri dishes, on plastic microtiter plates and in plastic culture flasks. Glass surfaces were washed with diethyl ether:ethanol (1:1) for 1 h, rinsed with distilled water and then with absolute ethanol for 1 h, and dried at 80°C overnight. Both plastic culture-ware and washed glass surfaces were treated with 15 μg/ml polylysine (Sigma) in water for 1 h, rinsed two times with water and then with culture medium.

Three culture media were tested: DMEM/F12 (Sigma) containing 10% FCS (Gibco), 20 mM KCl, 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 μM cytosine arabinoside (ara-C); DMEM/F12 containing B27 serum-free supplement (Gibco), 20 mM

TABLE 1
Embryo Yields from Affected and Normal Ewes

Animals	Ewes flushed	Corpus lutea per ewe mean (SD)	Embryo yield: Embryos recovered per donor	Embryo yield: Embryos frozen per donor	Recipient pregnancy rate (%)
Affected	29	8.0 (5.4)	3.1	3.0	42
Normal	5	8.2 (4.1)	4.8	3.8	61

KCl, penicillin, and streptomycin; and Neurobasal medium (Gibco) containing B27.

Petri dish and microtiter plate cultures were covered with a thin layer of silicone fluid (Dow Corning 50CS) and flasks with filtered vents were used (Nunc). A fungicide, Fungizone (Gibco), was routinely added the culture media in later experiments. Cells were incubated at 37°C in 5% CO_2 and maximum humidity. Half-volume media changes were made weekly. Cultures were monitored with Hoffman modulation contrast optics and photomicrographs were taken at regular intervals. Cell diameters were measured by comparison with human red blood cells that have a diameter of 7.5 μm.

Immunocytochemical identification of neurons. Coverslips were washed free of medium in DPBS and the cells were fixed for 5 min in 2% paraformaldehyde, treated with 0.03% H_2O_2 for 20 min, and then blocked with 1% sheep serum in PBS containing 0.3% Triton X-100 for 30 min. Mouse anti-bovine microtubuli-associated protein 2 (MAP2) antiserum, 50 μl (Boehringer), was pipetted onto each coverslip, overlaid with laboratory film, and incubated in a humidified chamber overnight at 4°C. After washing in PBS, the coverslips were similarly exposed to sheep anti-mouse IgG-peroxidase conjugate (Boehringer) for 2 h at room temperature. MAP2 was localized using diaminobenzidene (DAB, Boehringer).

RESULTS

The first step was a systematic investigation of the influence of fetal age on cultures. Control and affected ewes were superovulated and inseminated with semen from control and affected rams, and embryos were collected and stored frozen. They were then thawed later and transplanted into surrogate dams as required.

Initially 89 affected embryos suitable for freezing were recovered and 45 normal ones. A lower proportion of the affected embryos was viable after thawing and the pregnancy rate in ewes receiving Battens embryos was also lower than in those receiving normal embryos (Table 1).

Fetuses of gestational ages from 50 to 125 days were compared. Development of control and affected fetuses was the same. This was a period of exponential growth, from a fetus weight of 20 g at 50 days to 4 kg at 130 days. Brain weights increase proportionally, in line with the data of McIntosh *et al.* (15, Fig. 1). At 50 days the brains consist of two smooth-surfaced hemispheres and the cerebellum is clearly defined, but there is no obvious demarcation between gray and white matter. At 90 days the brains look like miniature adult brains, with invaginations and defined gray and white matter.

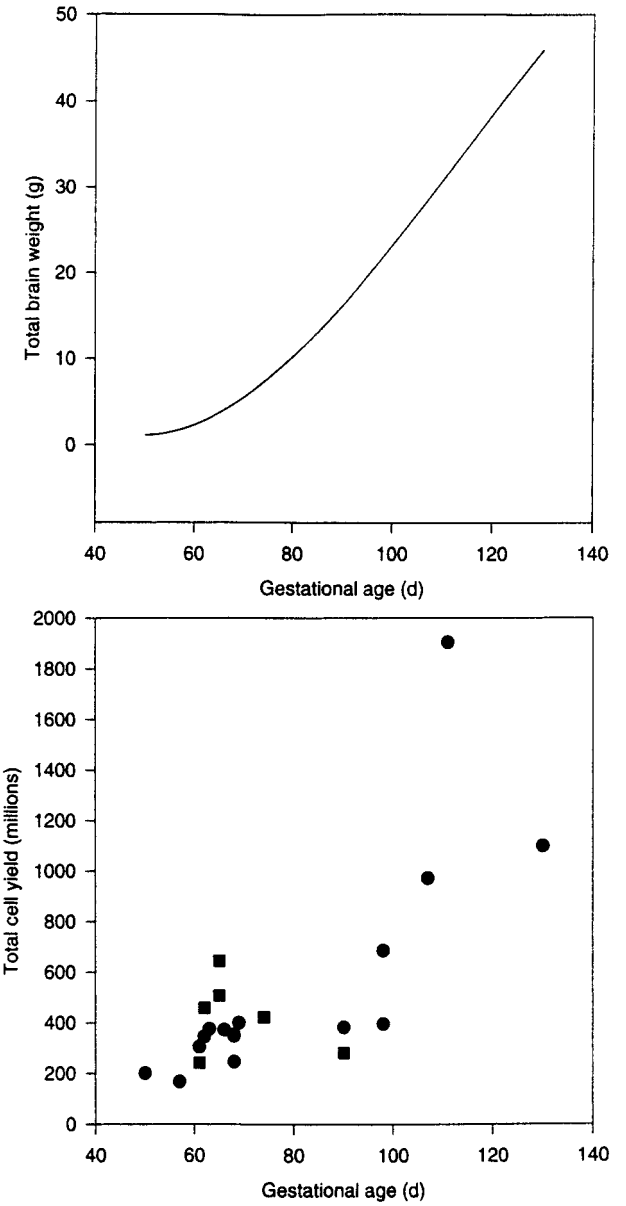

FIG. 1. (Top) Growth rates of sheep fetal brains (data from McIntosh *et al.*, 15). (Bottom) Yields of viable cells obtained from affected (■) and control (●) fetuses of different ages.

Despite the rapid growth no more cells were harvested from the older brains, up until 105 days. Mean yields from control and affected fetuses were

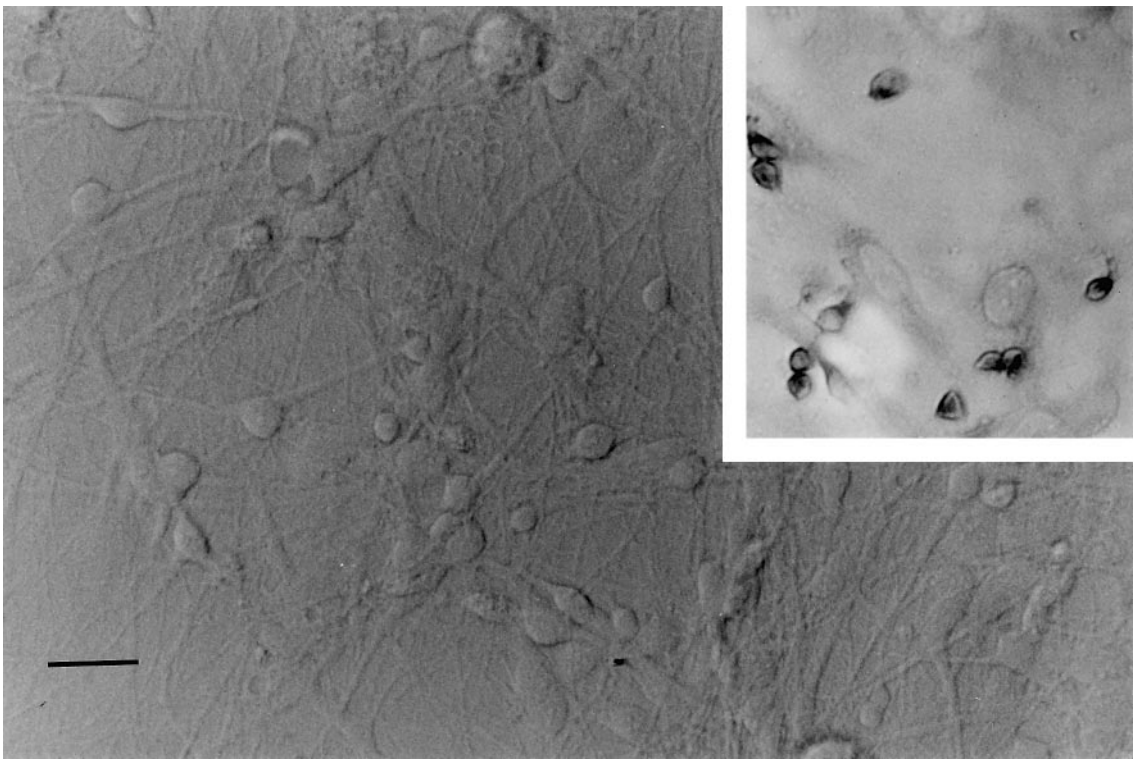

FIG. 2. Cells isolated from a 68-day-old fetus cultured in DMEM/F12 containing B27 for 35 days, visualized with Hoffman modulation contrast optics, and immunostained with MAP2 antiserum (inset). Bar represents 20 μm.

the same. Between 200 and 600 million viable cells were recovered from each fetus (Fig. 1).

Treatment of all surfaces for culture, in this case with polylysine, was essential for neuron adhesion. Of the media tested, DMEM/F12 with B27 was the most practical. Cell performance was similar in Neurobasal with B27, but this is more expensive. Cell viability was not as good in culture in medium containing FCS and the need for ara-C to control glial cells was an additional disadvantage.

No developmental or viability differences were observed between normal and affected neurons in culture. Fetuses from all ages up to 125 days yielded cells that plated down well and developed a network of neurites. At plating out, the cells were rounded. A day later single process outgrowths had begun. These grew rapidly and in 2 days some were at least 100 μm long. After 4 days they were over 200 μm. Neurites were long and slender with little branching. By Day 6 they had created a network (Fig. 2).

Most neurons were bipolar and a small proportion were multipolar, even after extended periods of culture (>90 days). The mean diameter of the bipolar cells increased slightly with time in culture, from 6.4 μm at 5 days to 7.1 μm at 70 days but no change was noted in the diameter of the multipolar cell bodies nor was the diameter influenced by the age of the fetuses. Normal and affected neurons were the same size. Neurons from fetuses in the 50–65 day age range persisted in culture for over 100 days while those from the 105 day group persisted for only 40 days.

Nonneuronal cells in media containing FCS multiplied rapidly but treatment with ara-C curbed proliferation. B27-based media (DMEM/F12 or Neurobasal) did not promote glial cell proliferation. Staining showed that neurons were often closely associated with glial cells. In the absence of glial cells, cell bodies and neurites attached directly to the glass or plastic polylysine-treated surface. With continued culture, these neurons became less firmly attached, often resulting in substantial loss of cells from the surface during processing for microscopy.

DISCUSSION

Short-term ovine neuron culture has been reported (16,17), but this is the first report of longer

term culture. The methods used are based on those for rodent neuron cultures and their application to sheep was quite straightforward. The only refinement, culturing under silicon oil, dramatically reduces the risk of fungal contamination and slows gas exchange, allowing longer times out of the incubator before the pH changes. The other difference is the relative fetal age, 60–100 days out of 150 days term in the sheep compared to 16–19 days out of 21 days in the rat. Little is known of prenatal brain development in sheep. As the extent of prenatal development varies greatly between species it is hard to compare these ages. Myelination, considered to be an indicator of the onset of neuronal differentiation, peaks at 120 days in the cerebrum and 155 days in the cerebellum (18,19). Neurons persisted mainly as simple bipolar cells even when cultured through to the equivalent age of a lamb at birth. These bipolar cells are of similar size to neurons from human fetus, 6.3 μm (20), and rat brain, 4.6 μm (21). Affected neurons are no more difficult to culture than control ones and their survival is similar under the conditions developed.

Our strategy of collecting embryos from superovulated ewes, prior to severe onset of the disease, and fetus development in normal recipient ewes at any time to suit experimental requirements, makes working with sheep neurons nearly as convenient as working with rodent cells. The large number of cells obtained from each sheep, over 200 million (Fig. 1), is a big advantage, as it allows a number of experiments to be done on one preparation. Work is underway to extend this advantage by freezing cells for later thawing and plating. Different culture milieu have been developed for different purposes, ranging from 50-μl drop cultures containing 15,000 cells under oil for assaying the effect of expensive growth factors to culture of 1,250,000 cells in flasks for subcellular fractionation studies.

Affected ewes responded to superovulation as well as normal ewes and the slightly greater loss of embryos observed during pregnancy is unlikely to be systematic. Unlike most domestic sheep the affected sheep are not subject to a constant active breeding program to maintain fertility. Affected embryos may be more sensitive to cryopreservation and alternative methods such as the Open Pulled Straw vitrification method, beneficial for *in vitro*-produced embryos (22), are being investigated.

Despite their normal growth and development affected neurons do accumulate subunit c in fluorescent storage bodies (23). Fortunately these cells persist in culture and do not reject the accumulating material. This *in vitro* model will be used to investigate a number of key issues including the kinetics of subunit c turnover in pulse-chase studies, and the possibility that subunit c participates in the formation of an ion pore (24,25) in patch clamp studies.

So far we have not observed cell death or morphological changes to equate with neurodegeneration. Some sort of interaction with other cells, or stress which could induce excitotoxicity or apoptosis, may be required. Differentiated neurons from neonatal dogs required growth factors to induce neurite development (26). Observable brain differentiation in sheep is beginning at 90 days fetal age (18,19) and we are currently exploring cultures from older fetuses. As soon as some equivalent of neurodegeneration is observed it will be used to explore the relationship between subunit c accumulation and neurodegeneration, and to test possible treatment regimens aimed at preventing neurodegeneration.

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