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Amniotic fluid cells and human stem cell research – a new connection

Andrea-Romana Prusa, Markus Hengstschläger

Department of Prenatal Diagnosis and Therapy, Obstetrics and Gynecology, University of Vienna, Vienna, Austria

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Summary

Currently it is the hope of both patients and investigators that human progenitor cells and stem cells can be widely used to replace dysfunctional cells within a tissue. It is speculated that such cells may prove to have the potential to treat or cure a myriad of diseases, including Parkinson's and Alzheimer's diseases, heart disease, diabetes, stroke, spinal cord injuries, and burns. A major goal in this area of research is to identify potential new sources for the isolation of progenitor cells or stem cells, without raising the ethical issues involved in embryonic stem cell research. Despite the widespread and well-established use of amniotic fluid cells in routine prenatal genetic testing, current knowledge about the origin and properties of these cells is limited. A wide variety of different origins has been suggested for the mixture of cells within amniotic fluid. Recent observations on cell cultures from amniotic fluid and on amniotic epithelial cells provide evidence that they may represent new sources for the isolation of cells with the potency to differentiate into different cell types. Are these cells suitable for use as primary cell systems for basic research? Do these cells provide a new source for research on stem cell biology? Can amniotic fluid cells be used to develop new approaches in tissue engineering? In this article the authors review the current state of knowledge about these cells, focusing on these questions.

key words: **amniotic fluid cells • human stem cells • amniocentesis**

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Author's address: Markus Hengstschläger, Department of Prenatal Diagnosis and Therapy, Obstetrics and Gynecology, University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria, email: markus.hengstschlaeger@akh-wien.ac.at

AMNIOTIC FLUID CELLS (AFCS)

AFCs are used for prenatal diagnosis of a wide range of fetal abnormalities caused by genetic mutations [1–3]. Although routine diagnosis using AFCs is well established and widely used, little is known about the origin and properties of these cells. Even in the case of pregnancies with normal fetal development, the cells in the amniotic fluid are heterogeneous. A wide variety of investigations performed since the 1980s have provided evidence that cells of all three germ layers (ectoderm, mesoderm and endoderm), depending on the gestational age, fetal pathology, etc. can be detected in human amniotic fluid. For specific subsets of amniotic fluid cells, the embryonic/fetal origins remain unclear. Furthermore, it has been suggested that both human amniotic epithelial cells (HAECs) and trophoblasts form part of the spectrum of cells in the amniotic fluid [1,2].

The average amniotic fluid volume has been found to be relatively constant: 207±92 ml at 16 weeks, 258±97 ml at 18 weeks, and 365±88 ml at 20 weeks [3]. On the other hand, the number of cells in second-trimester amniotic fluid varies between 10 and 1000 cells/µl. This spectrum of variation is even larger when pregnancies with fetal pathology are included, due to the effects of specific fetal anomalies on the number of AFCs (AR Prusa, M Rosner, E Marton, G Bernaschek, M Hengstschläger, unpublished observation).

What types of cells can be found in human amniotic fluid? In the course of AFC cultivation for routine genetic diagnosis, adhering and dividing colony-forming cells are selected. Accordingly, in the past scientific interest was focused on the characterization of these cells (see below). More generally, it is important to consider all types of AFCs (Figure 1): not only the adherent, dividing cells used in routine diagnosis, but also the cellular content of native amniotic fluid.

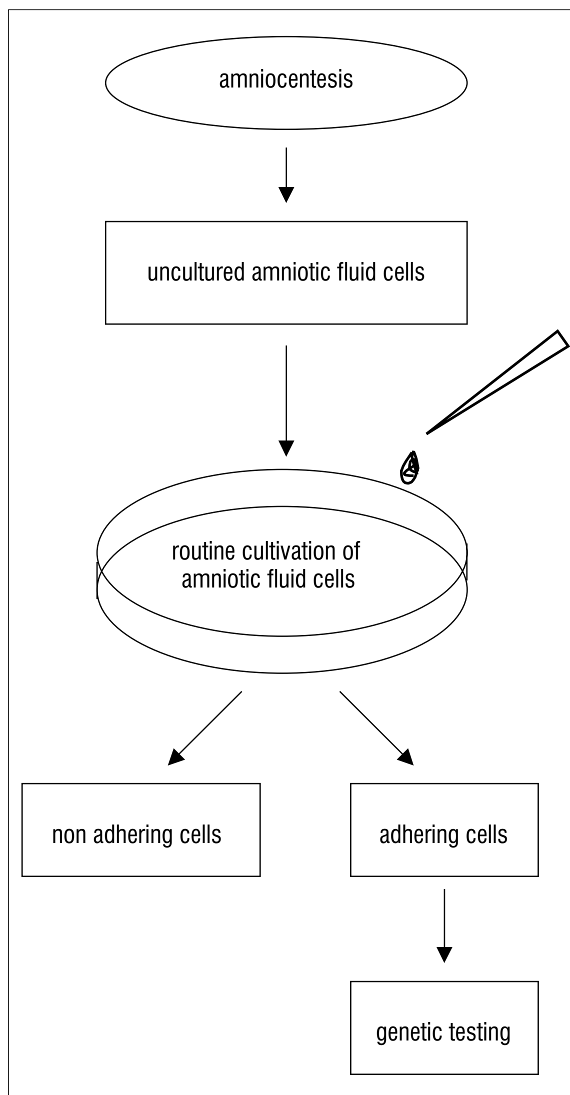

Figure 1. Human amniotic fluid cells used in routine prenatal genetic diagnosis.

It is clear that the vast majority of cells within native amniotic fluid are incapable of attachment and proliferation under the cell culture conditions used in routine prenatal laboratory diagnosis [1–3]. However, many of these cells are viable, as can be concluded from trypan blue exclusion assays. On the other hand, it is possible that specific AFCs have the capacity to attach, but do not proliferate or form colonies because of cell cycle arrest, differentiation status, or senescence. A general classification of AFCs should consider these different aspects.

The viable cell population in the amniotic fluid contains:

1) cells attaching under routine culture conditions:
   a) dividing, colony-forming cell types,
   b) cells which do not proliferate under routinely used culture conditions,

2) cells not attaching under routine culture conditions:

Currently, almost nothing is known about the cells described in 1b) or 2) above. It must be a major aim of future research on this topic to perform experiments to investigate the origin and properties of these cells. It is very likely that at least some specific subpopulations of these cells can be grown under cell culture conditions other than those used in routine prenatal laboratory diagnosis.

Moreover, we have only limited knowledge about the widely-used attaching, colony-forming AFCs. Early classifications of these cells were mainly based on morphological criteria, and are thus inadequate. Until now, only very limited biochemical data on these cells are available. Interestingly, it has been observed that no correlation can be found between the AFCs in the native fluid and the amount of colony-forming cells. Amniotic fluid cells from a normal fetus when plated out for *in vitro* culture adhere to glass or plastic surfaces comparatively slowly, in 3–4 days. Very importantly, it has been found that in cases of certain fetal abnormalities, including an open lesion, such as a neural tube defect, or an abdomi-

nal wall abnormality, such as gastroschisis, the morphology and growth properties of the cells in the amniotic fluid are different. Thus a general issue of relevance for further research on AFCs is that fetal anomalies have a significant impact on the spectrum of cells in the fluid [1,2].

A mixture of morphological aspects, limited biochemical criteria, and growth characteristics led to the classification of AFCs, which attach and form colonies under routine culture conditions, into three major groups:

- epitheloid E-type cells;
- amniotic fluid specific AF-type cells;
- fibroblastic F-type cells.

AF-type and E-type both appear at the beginning of cultivation. AF-type cells persist during the cultivation process, while E-type cells soon show a significant decrease. F-type cells cannot be cloned from every amniocentesis sample. They usually occur late during cultivation. Although it is believed that there exist as yet unknown additional origins for all three cell types, E-type cells have been thought to derive from fetal skin and urine, AF-type cells from fetal membranes and trophoblasts, and F-type cells from fibrous connective tissue and dermal fibroblasts [1,2]. AF-type cells produce estrogen, human chorionic gonadotropin and progesterone, which suggests that these cells originate from (placental) trophoblast tissue [4]. Also, due to the lack of hormone production, F-type cells are considered to originate from mesenchymal tissue, while both types express HLA Class I (HLA-ABC) surface antigens, but not HLA Class II (HLA-DR) [5].

HUMAN AMNIOTIC FLUID: DIFFERENTIATED CELLS, PROGENITOR CELLS, STEM CELLS

It is not difficult to imagine that differentiated cells of varying lineages are present in human amniotic fluid. Cells belonging to the amnion, skin, and the urogenital, respiratory, and digestive systems can be found. Furthermore, depending on the occurrence of fetal anomalies, the spectrum of cell types varies, including different neuronal cells. Accordingly, it is tempting to speculate that by using specific cell culture conditions amniotic fluid can be made an important source for studies on primary (non-transformed, non-immortalized) human cell types. For example, infection of AFCs with a retrovirus vector containing MyoD, a gene regulating myogenesis, induces muscle differentiation with formation of myotubes and expression of specific proteins, such as dystrophin [6].

Telomerase is an enzyme which adds telomere repeats onto chromosome ends to overcome the end replication problem. Telomerase activity is detectable in human pluripotent stem cells, in germ cells, in most immortalized cell lines, and in tumor samples. Amniotic fluid does not contain immortalized or tumor cells, but even so telomerase activity has been found in both uncultured and cultured AFCs at 14 weeks of gestation [7]. Further investigations are necessary to clarify which cell types within the mixture of AFCs express high telomerase activity.

Human amniotic epithelial cells (HAECs) constitute the inner layer of the amnion and are formed from the amnioblast on the eighth day after fertilization. It has long been proposed that HAECs could harbor the potential to differentiate into a wide variety of different organs, including heart, liver and brain [8]. At least under certain circumstances, HAECs are believed to be part of the cellular content of amniotic fluid. Strikingly, it has been demonstrated that HAECs express markers of glial and neuronal stem cells [9]. Furthermore, evidence has been found for the synthesis and release of acetylcholine [10] and catecholamines [11] by HAECs. The presence of acetylcholine and catecholamine has also been demonstrated in amniotic fluid and in amniotic fluid cells [12]. In addition, HAECs and human amniotic fluid have both been demonstrated to express neurotrophic factors: nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 [13,14]. Whether this is due to HAECs in the amniotic fluid or whether other cell types within the fluid also express these markers remains to be clarified.

These data prompted the authors to further investigate whether HAECs could represent a potential donor source for transplantation therapy. Dissociated HAECs were transfected with the *Escherichia coli* LacZ marker gene and implanted into the previously dopamine-depleted striatum of immunosuppressed rats. Two weeks post-grafting, HAECs were demonstrated to have survived without overgrowth, as evidenced by marker gene-positive cells. The experiments described in this study [15] provide evidence that HAECs capable of producing dopamine can survive and function in the brain of a rat model of Parkinson's disease. Further research in this direction must be aimed at enhancing the ability of HAECs to produce dopamine and to improve the conditions for graft survival [15]. In any event, these are very encouraging data, stressing the importance of further investigations.

TISSUE ENGINEERING

A very interesting new study was aimed at determining whether fetal tissue constructs can be engineered from cells in amniotic fluid. AFCs from pregnant ewes were grown in culture, and a subpopulation of morphologically distinct cells was mechanically isolated and selectively expanded under specific culture conditions. Immunocytochemically these cells stained positive for vimentin, smooth muscle actin (SMA), cytokeratin 8 and 18, and fibroblast surface protein (FSP), and negatively for desmin and cluster differentiation 31 (CD-31). This profile is consistent with a mesenchymal, fibroblast/myofibroblast cell lineage. Another important finding was that these cells exhibited significantly faster proliferation than comparable fetal and adult cells. After expansion, these cells from the amniotic fluid were seeded onto a polyglycolic acid polymer/poly-4-hydroxybutyrate scaffold. The resulting construct was analyzed by both optical and scanning electron microscopy. Scanning

electron microscopy revealed dense, confluent layers of cells surrounding the polymer matrices and firm cell adhesion of both scaffolds. No cell death was observed [16].

This is important evidence that amniotic fluid can serve as a source of cells for fetal tissue engineering. Given the mesenchymal lineage of the cells cultivated under these specific conditions, such a construct could have applications in a variety of congenital anomalies. A scenario could be imagined that ultrasound examination detects congenital diaphragmatic hernia or body wall defects. Therefore, amniocentesis is performed to enable detection of putative genetic alterations. A cell construct is cultivated from AFCs. This engineered construct could be important, as surgeons may need the tissue for use as a patch to repair the congenital defect. It has been speculated that a 2 ml sample of amniotic fluid could yield enough cells to create such an engineered construct – material containing cells from the fetus, to function as a graft – for implantation immediately after birth. Following up on these findings, it would be interesting and important to determine whether cell lines other than those described in this study can be expanded from amniotic fluid specimens. This is certainly dependent on the cultivation conditions used for expansion. Another important question is whether fetal pathologies can lead to the availability of cells not normally found in healthy pregnancies [16,17].

CONCLUSIONS

Much of the recent excitement surrounding human progenitor cells and stem cells is due to their potential use for replacing dysfunctional cells within a tissue. It is the hope of investigators and patients alike that such cells will have the potential to treat or cure a myriad of diseases, including Parkinson's and Alzheimer's disease, as well as heart disease, diabetes, stroke, spinal cord injuries, and burns [18]. The fact that the most potent stem cells are probably those derived from the inner cell mass during embryonic development raises a number of ethical issues [19]. On the other hand, recent investigations provide evidence that other progenitor cells or stem cells, isolated from a variety of human sources, may also have the potency to differentiate into a wide spectrum of different cell types. One widely discussed source for such stem cells is fetal cord blood. Concerning these questions, we believe that the findings discussed above warrant further investigation of cells in the amniotic fluid, human amniotic epithelial cells, and probably also placenta cells (Figure 2):

1) To obtain a rough idea of the different cell types present in amniotic fluid, we suggest performing an RT-PCR screen for the expression of known marker genes for progenitor cells, stem cells and differentiated cell types.

2) Such an RT-PCR screen could lead to the establishment of enrichment and isolation protocols based on protein expression detection and flow cytometry.

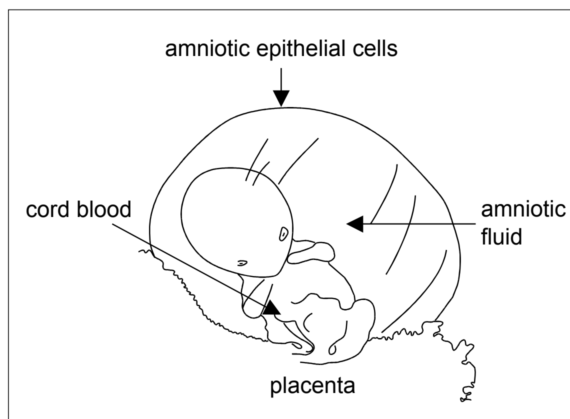

Figure 2. Interesting sources of different cell types of early human origin.

3) Enrichment of specific cells from amniotic fluid could also be performed by employing specific cultivation conditions. Protocols need to be established and tested to enable the cultivation of other cell types derived from amniotic fluid in either normal or pathological pregnancies.

4) In addition, it would be of great interest to determine whether specific cell differentiations, induced by known differentiation media, can be obtained with AFCs.

5) If a specific cell type or cell lineage is characterized and enriched, additional approaches could allow for further identification: On the single cell level, mRNA profiling via PCR techniques could be performed. If an appropriate amount of cells can be enriched, even microarray or proteomics approaches could be employed.

6) Cells isolated from amniotic fluid could further be injected into rodents to identify their targets within the body and to investigate whether they can fulfil functions at their target organs.

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