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Characteristics of cultivated cells from amniotic fluid and culture failures.

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Characteristics of cultivated amniotic fluid cells have been studied. Three main types of cell are classified; fibroblast like cells, epithelioid cells and intermediate type cells. Failures of culture caused by many crucial factors are also discussed.

Key word : Amniotic fluid cells, Culture failures, Cultivated aminiotic fluid cells

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เซลล์น้ำคร่ำที่ได้จากผู้ที่มีอายุครรภ์ 15-22 สัปดาห์ เมื่อนำมาเพาะเลี้ยงในห้องปฏิบัติการโดยใช้น้ำยาเลี้ยงเซลล์ 2 ชนิด คือ McCoy 5A และ Chang medium ปรากฏว่าเซลล์ที่เพาะเลี้ยงได้จะมีอยู่ 3 ชนิดใหญ่ ๆ คือ (1) เซลล์คล้ายไฟโบรบลาสท์, (2) เซลล์คล้ายเซลล์บุผิว และ (3) เซลล์ที่มีลักษณะก้ำกึ่งระหว่าง (1) และ (2) นอกจากนี้ยังพบเซลล์ที่มีรูปร่างเป็นอย่างอื่นอีกมาก

ส่วนความเหมาะสมของน้ำยาที่ใช้เพาะเลี้ยงนั้น Chang medium ดีกว่า McCoy 5A เพราะให้ปริมาณและการเจริญเติบโตของเซลล์ดีกว่า

Nowadays, amniocentesis under ultrasonography is an advance, prenatal diagnosis, developed for routine clinical practice. Cytogenetic diseases can be diagnosed from 15-16 wks. gestation.[1] This diagnosis employs cells originating from amnion, fetal skin, part of digestive tract, respiratory tract and vagina seed in culture vessels.[2] Cultivated amniotic fluid cell morphology can be used for interpretation the successful of prenatal diagnosis. Miller, et al[3] had succeeded in using the amniotic fluid cell morphology in prenatal genetic diagnosis. However, characteristic of cultivated amniotic fluid cell grown in culture medium are basis for many of these diagnostic test. Some one may not be familiar with the basic laboratory procedure which underlie the detection of fetal abnormalities. Also, problems about cell growth, types of cell that cause the antenatal cytogenetic analysis failure are always being faced. The purpose of the present study is the analysis of various characteristics of cultivated amniotic fluid cells in primary culture, culture failures and basic media for successful all growing and shorter culture time in providing faster diagnostic results.

Materials and methods

Twenty milliliters of amniotic fluid obtained from routine transabdominal amniocentesis from pregnant women, who come to the antenatal clinic at the Department of Obstetric and Gynecology, Chulalongkorn Hospital. Patients have been adviced by Obstetricians concerning possible risks in amniocentesis. The gestation period varied between 15-22 wks. and criterias for amniocontesis depended on the opinion of obstetrician, such as advanced maternal age (>36 years) or previous child with cytogenetic diseases, etc; the number of the sample was about 100 cases. Of obtained amniotic fluid, 80% were clear and straw colour, with no contamination of red blood cells. The fluids were equally divided into 2 centrifuge tubes (10 ml fluid in each), firmly closed with screw cap and then centrifuged at 800 rpm for 10 min. The supernatant was decanted for alfa-feto protein study. The pellet cells were resuspended gently in the remaining 1 ml fluid in centrifuged tube and seeded in two 50 ml plastic vessels (cel-cult) which contained 2 different types of cultured media. One was 5 ml. cultured medium containing 80% modified McCoy 5 A (Gibco), 20% fetal calf serum (FCS) (Gibco), penicillin 10,000 iu/ml, streptomycin 10,000 ug/ml and fungisone (Gibco). The other one had 5 ml. of Chang medium (HANA) (1 part of Chang C+9 parts of Chang B), glutamine 200 mM and antibiotics and fungisone in the same concentration. Processes of seeding were done in the UV-lamp hood and lamina air flow cabinet. Before culture initiation morphologies of amniotic fluid cells were checked by using criteria of Steele and Breg[4] that is two main types of cell: (a) large cell characterized by irregular border and small nucleus, causing them to resemble squamous epithelial cell (epithelial-like) and (b) small round or oval, smooth borderd cell (macrophage-like)

The cultured vessels were kept in incubator at 37°C and a 5% co_2 atmosphere is constantly maintained. The first checking of cell growth were done on the 6th or 7th day after initiation and the first medium change were done later. On the next day characteristics of cell growth and forming colony were recorded. If there was no visible cell or few-cell growth or no visible colony by 7-10 days, it was difined as slow growth or no growth (culture failure) and micro-organism contamination was also classified as failure.

Results

Characteristics of amniotic fluid cells

On examination of the cellular morphologies, two main types were found, that was about 90% were epithelial-like cells and 10% were macrophage-like cells.

Characteristics of cultivated amniotic fluid cells

cellular morphology of amniotic fluid cells was difficult to classify into distinct types. However, There were 3 main types appearing in the culture: (a) fibroblast-like cell (b) epithelioid cell and (c) intermediate cell type or amniotic fluid cell. After seeding, cells started as singly dispersed cells, and 24 hr. after culture initiation, cells started to grow, especially spindle-shape cell were laid down. The lace-like network of fibroblasts appeared about 6-7 days of culture and the network will eventually be filled by proliferating cell to form solid colony (Fig 1). The epithelioid cells formed small solid colonies and enlarged and do not generally grow to confluency. For the intermediate cell type, there were two main morphologies: large, flattened cell and small, more rounded than fibroblast-like cell. Growth of these two cells were slow and not confluent as fibroblast-like cells. In our laboratory epithelioid cell type predominated as the fibroblast-like cell was less seen in Chang medium. In both types of media one colony may contain more than one type of cells. Beside the three main types of growing cell, heterogeneous morphologies of cell are also noted; large cell with many processes looked like neuron, fibroblast-like

cell with very long spindly processes, very large epithelial-like cell with ruffled border, etc. (Fig. 2)

The number of primary colonies varied from 0-8 per culture flask and the size of colony varied 1-4 mm in diameter in modifed McCoy 5A medium and the rate of growth was slow compared to growth in Chang medium.

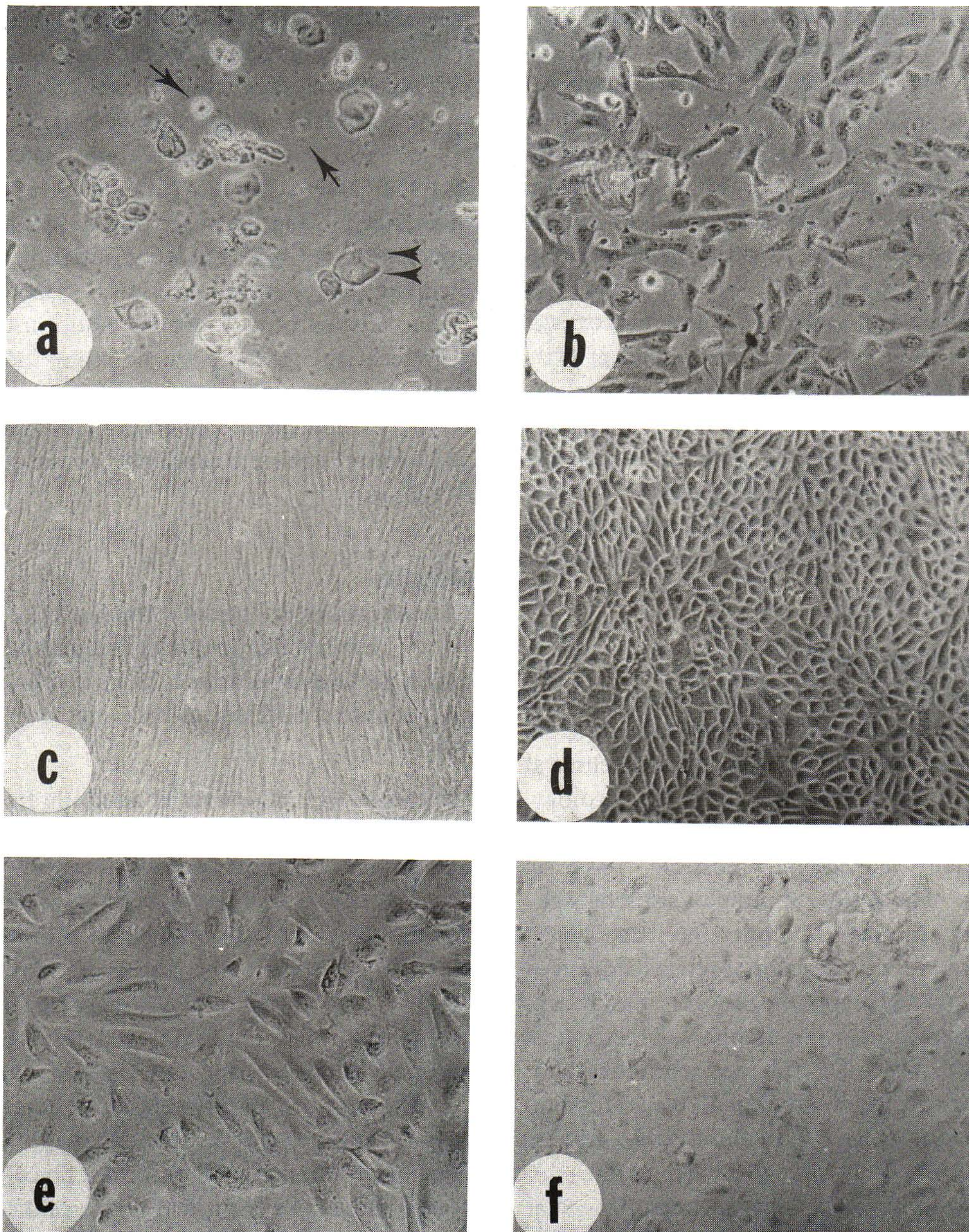

Figure 1. Morphological idendification of human fetal cells commonly seen in primary culture.

(a) single dispersed amniotic fluid cell after seeding, large cell irregular border and small nucleus, resemble to squamous epithelial cell. (double arrow head) and small, round or oval, smooth border cell (arrow).

(b) lacelike network of fibroblast after 6-7 day seeding.

(c) confluent colony of fibroblast-like cells.

(d) actively colony of epithelioid cells.

(e) intermediate cell type, large & flattened cells and

(f) intermediate cell type, small, less spindly and more round than fibroblast like cell. (All phase contrast microscopy $\times$ 600).

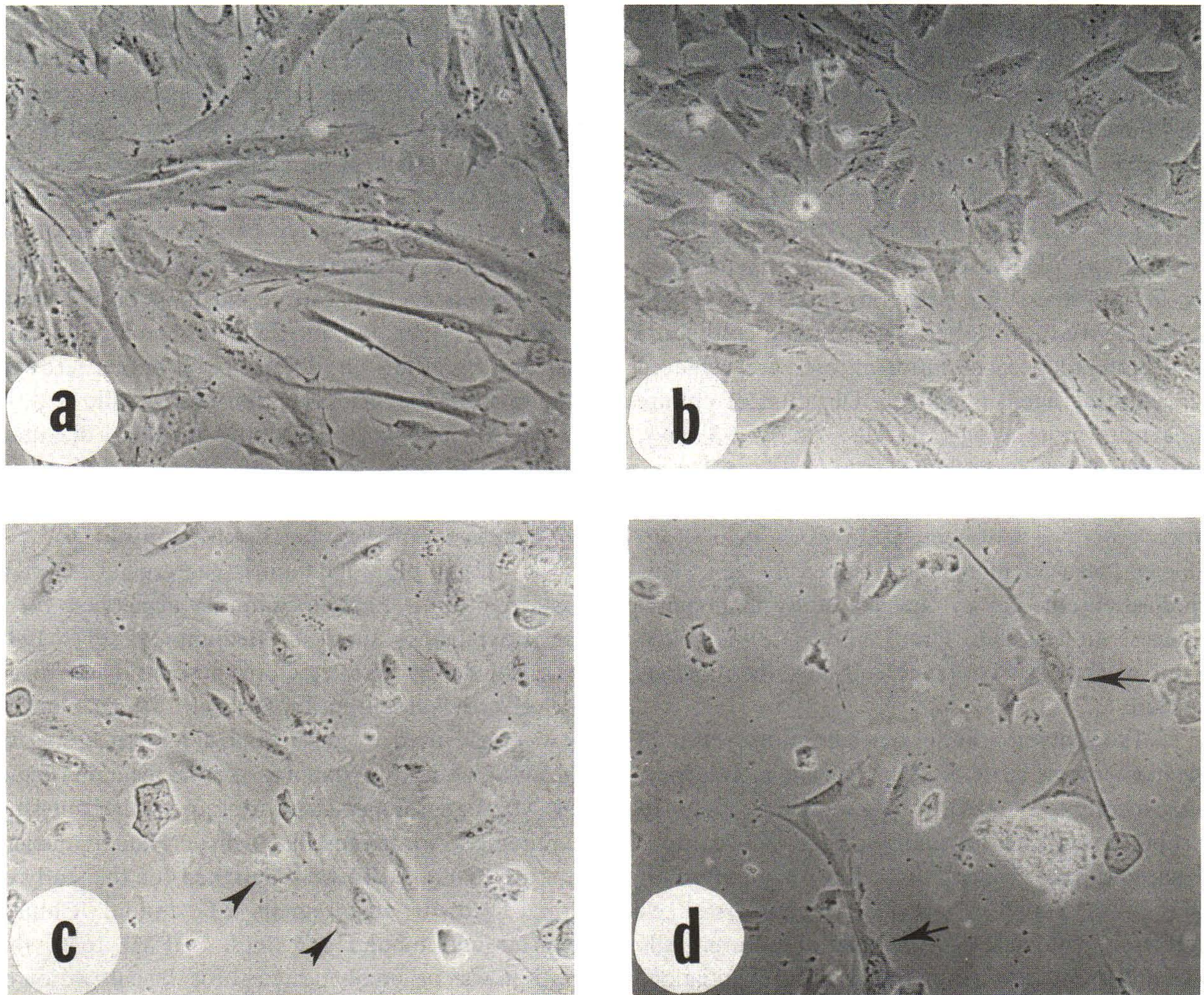

Figure 2. Heterogenous morphologies of cultivated amniotic fluid cells.
(a) large, elongated projection and ruffled cells.
(b) small, short spindly projection and urffled cells.
(c) large squamous cell and ruffled border cell (arrow head) and
(d) cell with long processes look like neuron (arrow) (All phase contrast microscopy × 600).

Growth failures

Percentage of both failure types were about 6.8 percents in McCoy 5 A medium; most of them had of no growth or slow growth.

Discussion

Characteristics of cells in the amniotic fluid

Category of cells in amniotic fluid have been defined by morphologies criteria, two main types have been described. However, Casadei et al[2] had identified these two main types into five morphological variants of amniotic fluid cells. These two main types of cell predominated in samples obtained at 14-20 wks. gestation.[2,5] Martin[6] had analysed 100 cells from each of amniotic fluid sample obtained at 16-37 wks. of gestation, indicating that 80-93 percent of cell scored were epithelial-like and the remainding 7-20% were macrophage-like, which corresponded to findings in our laboratory; about 90 percent were epithelial-like cells.

Characteristics of cultivated amniotic fluid cells

Cells in tissue culture are difficult to classify into distinct type because they form a morphological spectrum. Gerbie et al,[7] had classified cell growth into 2 basic types (a) fibroblast-like cell, resembling fibroblast and have long spindly projection and (b) epithelial-like cell, more compact and having no projection. Some authors recognized three types by adding the intermediate cell type or amniotic fluid

cell, a cell having intermediate morphology, elongated and have two pointed ends, yet they are less spindly, and more rounded than fibroblast.[8] Chang, H.C., et al[9] have described many characteristics of human fetal cells. For fibroblast and epithelial-like cells have no problem for classification. The intermediate cell type or amniotdic fluid cell is controversial in classification. However, different cell types may vary in usefulness for prenatal diagnosis.[5] For cytogenetic study, fibroblast-like cell and epithelioid cell is prefered because more mitoses are achieved after arresting agent is added. Duration of culture also affects morphology of cells.[5] By 2-3 days, loose cellular networks are usually visible, 6-7 days, colonies forming are seen and after the first medium change, growth is confluent, colony forming is cleary visible and also cell morphology are easily seen. This period is suitable for cytogenetic study in prenatal diagnosis. In long duration of culture, cell is not fit for this work because of cell death and spontaneous mutation.

From observation in our laboratory, cultured medium is also one factor in cell growth and morphology. Chang medium (HANA), a new medium developed for rapid growth of the amniotic fluid cells culture, can support proliferation of cell with good result such as in starting growth, increasing numbers of cell and forming colonies, and the yield of cultured cells can be utilized in short period (4-6 days) after initiation. The morphology is predominantly actively epithelioid cells, while fibroblasts and amniotic fluid cells are less seen. If modified McCoy 5A is used, less growth, and epithelioid and fibroblasts are observed. This means that Chang medium (HANA) is composed of nutrients suitable for epithelioid cell growth.

Culture failures

Culture failures are classified into two main types; (a) concerning cell growth and (b) microorganism contaminations. The main causes of failure of growth are from 3 crucial factors: (I) amniotic fluid cells, (II) serum and (III) the basic media. The problems of cell may be: (a) no viable cells present in the sample. This becomes more of a problem with late pregnancy; amniocentesis in late pregnancy reduce viable cells. In the case of amniotic fluid, the late transportation over long distances kept in not proper condition, viable cells are also reduced. (b) not enough cells in specimen, especially in samples taken before 16 weeks gestation. (c) heavily blood stained sample, large numbers of erythrocytes present prevent amniotic fluid cells from setting on the surface of vessels and (d) brown sample, this sample is usually full of dead cell debris and may be difficult to grow.[10]

As mentioned above, serum, the essential factor in composition of cultured media for cell growth has influence on culture failure. Amniotic fluid cell cultures also required fetal calf serum for growth. Great variation in the composition of commercially prepared lot and growth promoting effectiveness of fetal calf serum have been demonstrated.[11, 12] In amniotic fluid and human fibroblast growth, variation in ability to promote growth, among serum batches has been found by Milo et al,[13] so that some laboratories doing antenatal diagnosis prefer to identify batches of serum with superior growth promoting ability, and to order and freeze large amounts of these batches for routine use.

Using basic medium is one crucial factor in tissue culture. Each medium is developed for a specific purpose such as Medium 199 (TC 199), originally developed to study the nutrient requirements for cell survival and multiplication. This medium is now widely used to culture most human tissue for chromosome study. Minimal essential medium (Eagle) (MEM) was formulated later to study further nutrients requirement of mammalial cells. Ham's nutrient mixture F10 and F12 were designed for the study of clonal growth requirement of diploid Chinese hamster and human cell lines. RPMI 1640, this medium was originally designed for the cultivation of leukemic cell and long term culture of peripheral blood lymphocytes. Leibovitz L-15 was designed for the growth of human fibroblasts and Chang medium has been recently formulated for the rapid growth of the amniotic fluid cell culture and chorionic villus culture method.[10]

In former time, in our laboratory, modified McCoy 5A was routinely used for supporting amniotic cell growth because Chang medium was not available and culture failure was 6.8 percents which was higher than in Europe (2 percents failure)[9] while the basic medium (HAM F10) and serum used were also different from our laboratory. However, culture failures were always faced and now this problem can be overcome by the substitution of original medium by Chang medium. The good benefit of Chang medium is promoting cell growth in short duration; quantity and quality of cell growth are satisfactory. The disadvantages of this medium are short shelt life (6 months) and expensiveness.

Failure from bacterial or fungal contamination is also a problem; culture failure by these causes is less than failure from cell growth. Elimination can

be done if sterilization is well done. Laboratory works should be done on lamina air flow cabinet. Cautions in method of collecting amniotic fluid, glasswares sterilization, process of seeding and incubator in perfect condition, can prevent failure from these contaminations.

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