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Characteristics of human infant primary fibroblast cultures from Achilles tendons removed post-mortem

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ABSTRACT

Primary cell cultures were investigated as a tool for molecular diagnostics in a forensic setting. Fibroblast cultures had been established from human Achilles tendon resected at autopsies, from cases of sudden infant death syndrome and control infants who died in traumatic events ($n = 41$). After isolation of primary cultures cells were stored at −135 °C, and re-established up to 15 years later for experimental intervention. Growth characteristics in cultures were evaluated in relation to the age of the donor, the post mortem interval before sampling, and the storage interval of cells before entry into the study. High interpersonal variation in growth rates and cell doubling time was seen, but no statistically significant differences were found with increasing age of the donor (mean 19 weeks), length of post-mortem interval prior to sampling (6–100 h), or increase in years of storage. Fibroblast cultures established from post-mortem tissue are renewable sources of biological material; they can be the foundation for genetic, metabolic and other functional studies and thus constitute a valuable tool for molecular and pathophysiological investigations in biomedical and forensic sciences.

1. Introduction

Fibroblast primary cultures can be outgrown from biopsies of skin or ligaments [1–3]. Tendon fibroblasts are designated tenoblasts or tenocytes and show capability of maintaining viability post mortem [4–6]. Cultured fibroblasts are initially widened and highly mobile, then shift into elongated shape with a parallel pattern, as they become closer [1,7]. Cell division is limited by contact inhibition, if diluted; the fibroblasts divide again. According to the original theory by Hayflick [8,9], cells have potential to divide approx. 50 times before going into senescence [9–12], and the timespan depend on biological age [10,13]. Senescent cells remain viable [14], but show change in morphology [8,15,16] and metabolism [17]. Proliferation arrest depends on the telomere shortening rate [18], as a consequence of age [19,20], but can be induced in young cells by several factors, including oxidative stress [21–23], which has been shown in a study of human fibroblasts [18,24]. No clear age dependent proliferation rate has been found, but fetal fibroblasts show higher rates than cells from adult donors [1,25–27]. However, the growth characteristics of infant-derived fibroblasts are still unclear since few studies of infant cells have been published. Cells established from autopsy material are rarely used in forensic research [28–30], but fibroblast may be a useful tool especially in analyses of RNA, sensitive to post-mortem changes [31,32]. This paper presents a study of primary fibroblast cultures established from Achilles tendons. Proliferation was studied and related to the age of the donor, post mortem interval before sampling, and the storage interval before use.

2. Materials and methods

2.1. Case sampling

Children included in the study were examined and described in a parallel study of gene expression after heat stress exposure [33]. 30 SIDS cases were compared to 11 children (control group) who died in a traumatic incident with a well-defined cause of death. SIDS was defined according to the Nordic criteria [34]. Information on previous history and circumstances of death was obtained from forensic documents, police reports, and hospital records. Age of the

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child at the time of death, post-mortem intervals (interval from time of death, or found dead, until autopsy; reflects the time after the body is found) and storage time (represents the number of years from the establishment of the primary culture to the time of re-establishment prior to the present experiments) were estimated.

2.2. Cell sampling and culturing

Achilles tendon biopsies (approx. 1 cm) were removed aseptically during autopsy. Outgrowth and isolation of fibroblasts was performed in standard RPMI medium with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.29 mg/ml l-glutamine, in humidified incubators at 37 °C and 5% CO_2. Confluence state was regularly evaluated by phase-contrast microscopy, to avoid contact inhibition of growth. During passage (sub-culturing) the growth media was removed and the cells washed three times with phosphate buffered saline (PBS). Trypsin-EDTA solution 0.5% was added for 1–5 min at 37 °C to detach the cells. Following centrifugation at 1500 rpm for 3 min at room temperature cells were split (typically 1:2–1:4) and transferred to sterile culture flasks. Following establishment of the initial culture, aliquots of cells were frozen gradually to −70 °C and then transferred to −135 °C. Information on the initial isolation process did not include information on growth rates, but they had all been sub-cultured 2–4 times before the experiment started.

2.3. Cell count

Growth was studied over 50 days with initial seeding of 3×10^5 cells and automated cell counting (NucleoCounter NC-100) every 10th day. Measurement error in cell counting was investigated by ten repeated measurements of three different cell densities (low (approx. 10.000 cells/ml), medium (approx. 100.000 cells/ml), and high (approx. 250.000 cells/ml)). Results were mean: 11,111, SD: 1054.093 for low density, mean 100,111, SD: 7373 for medium density, and mean 252,889, SD: 24,132 for high density. The coefficient of variance (machine error plus variation in sample handling) was thus 9.5, 7.4, and 9.5% for low, medium, and high densities, respectively. Cell growth was evaluated by cell doubling time using the formula $N(t) = C(2)^{t/d}$, adapted after Roth [35]. ($N(t)$ = the number of objects at time t, d = doubling period (time it takes for object to double in number), c = initial number of objects, t = time). The doubling time (in days) = $d^*\ln(2)/\ln(c2/c1)$ was estimated (cell concentration at the time they were seeded out: $c1$, d = days in growth, cell concentration when harvested and sub cultured: $c2$). Data were analyzed in Stata 10.1 and Epidata 3.1 was used for the handling of data collected. P values less than 0.05 were considered to be significant. Data were skewed according to the normal distribution and log-transformation was used to display data on graph and nonparametric tests were used when appropriate, otherwise the data were analyzed with regression analyses.

3. Results

The proliferation of the 41 established fibroblast cell lines varied considerably between individuals (see Appendix). Cell growth was evaluated by estimating the cell doubling time (CDT) (see Section 2). The highest growth rate was observed in one of the SIDS cell lines and the growth rate was higher in the SIDS group (mean of 35,285 cell increase per day) than in the control group (traumatic incidents) (mean of 27,461 cell increase per day), although the observed differences were non-significant ($p = 0.18$). Mean cell doubling time was highly variable (Fig. 1); median of all individuals was 33, and 45 for controls and 30 for SIDS, but the differences between groups were non-significant ($p = 0.10$, Mann–Whitney). SIDS cases and infants who died in a traumatic event are

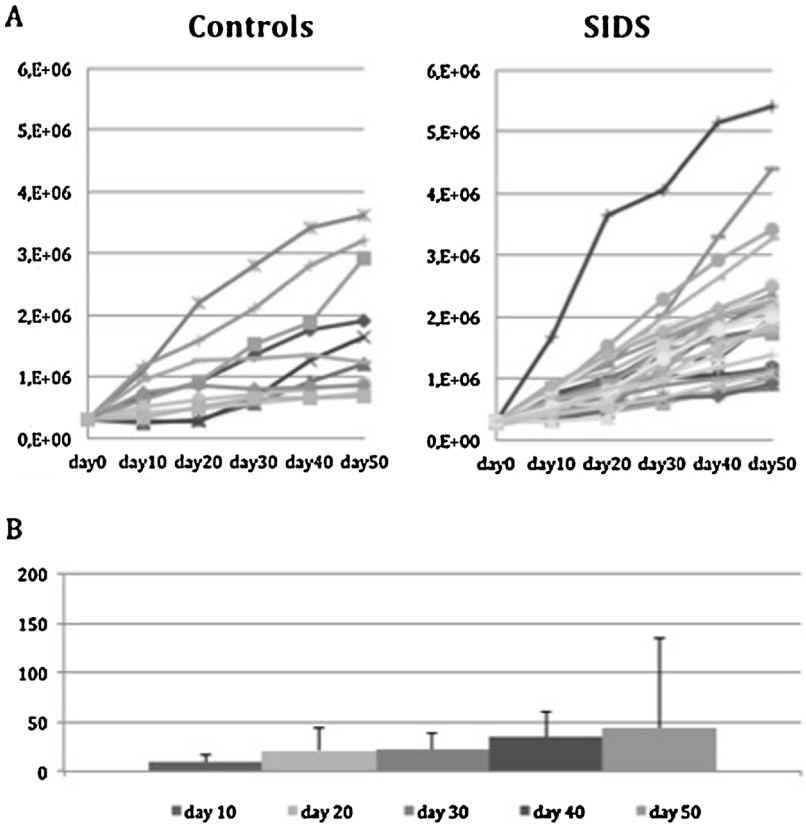

Fig. 1. SIDS and controls displayed at day 10, 20, 30, 40, and 50 of cell growth. (A) Single cases cumulated numbers displayed as curves. The x-axis refers to absolute numbers of cells count. (B) Median of cell doubling times for both groups. The numbers of the x-axis refers to the median of the cell doubling times of cells counted on the specific day.