Weierstraß-Institut
für Angewandte Analysis und Stochastik

im Forschungsverbund Berlin e.V.

Preprint ISSN 0946 – 8633

Experimental and Mathematical study of the influence of growth factors on the growth kinetics of adult human articular chondrocytes

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submitted: December 8, 2004

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No. 988
Berlin 2004

1991 *Mathematics Subject Classification.* 92D25, 92C37.

Key words and phrases. chondrocytes, cell expansion, growth kinetic, delay model.

This work was supported financially by the Swiss National Science Foundation, Research Program 46 *Implants and Transplants* (grant #4046-58623).

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Abstract

This study aimed at determining how kinetic parameters of adult human articular chondrocytes (AHAC) growth are modulated by the growth factor combination TGFβ1, FGF-2, and PDGF BB (TFP), recently shown to stimulate AHAC proliferation. AHAC, isolated from cartilage biopsies of three individuals, were cultured in medium without (CTR) or with TFP. For growth curves, AHAC were seeded at 1000 cells/cm^2 and cultured for 12 days, with cell numbers measured fluorimetrically in the same wells every 12 hours. For microcolony tests, AHAC were seeded at 2.5 cells/cm^2 and cultured for 6 days, with cell numbers determined for each microcolony by phase contrast microscopy every 8 hours. A mathematical model combining delay and logistic equations was developed to capture the growth kinetic parameters and to enable the description of the complete growth process of the cell culture. As compared to CTR medium, the presence of TFP increased the number of cells/well starting from the fifth day of culture, and a 4-fold larger cell number was reached at confluency. For single microcolonies, TFP reduced the time for the first cell division by 26.6%, the time for subsequent cell divisions (generation time) by 16.8%, and the percentage of quiescent cells by 42.5%. The mathematical model fitted well the experimental data of the growth kinetic. Finally, using both microcolony tests and the mathematical model, we determined that prolonged cell expansion induces an enrichment of AHAC with shorter first division time, but not of those with shorter generation time.

1 Introduction

In several cell therapy applications, the use of cytokines during cell expansion has been proposed as a promising method to increase the number of cells that can be obtained starting from a small biopsy, particularly for cell types with limited proliferative capacity (Beattie et al., 1997; Carpenter et al., 1999; Pittelkow et al., 1993; Simmons and Haylock, 1995; Stewart et al., 2003). Depending on the cell system under investigation, the cytokine-induced increase in cell number may underlay a variety of causes, such as a shorter time to start the first cell division, a shorter cell division time, a lower percentage of quiescent cells, and/or a larger density of cells reached at confluency. For example, Deenick *et al.* (Deenick et al., 2003) showed that IL-2 influences T-cell proliferation by increasing the proportion of cells that enter the first division and by reducing the average division time, but not by altering the time at which the cells enter the first division. In another study, Deasy *et al.* (Deasy et al., 2002) observed that specific growth factors (i.e., FGF-2, EGF,

SCF or IGF-1) induced proliferation of muscle-derived stem cell by recruitment into the cell cycle in case of freshly isolated cells, or by reducing the length of the cell cycle in case of an expanded cell clone. Thus, they demonstrate the importance of monitoring several parameters of cell growth following stimulation with growth factors.
Quantifying the appropriate kinetic parameters may also be relevant to investigate whether the effect of cytokines is related to a possible selection of certain subpopulations, and to develop realistic mathematical models characterising and predicting cell growth.
The most simple and frequently used mathematical models apply equations of exponential growth to estimate the population doubling time. The key assumption of these models is that all the cells in culture divide at the same time; therefore, the estimated doubling time reflects a macroscopic feature of the cell culture, which does not take into account the properties of individual cells. Non-exponential time-lag models have been shown to overcome these limitations and provide more realistic estimation of several parameters of cell growth kinetics (Baker et al., 1998; Sherley et al., 1995). However, to our knowledge these models have not yet been combined with logistic equations to model contact inhibition upon cell confluency.
Recently we reported that the number of adult human articular chondrocytes (AHAC) obtained following monolayer culture is markedly increased by the use of TGFβ1, FGF-2, or PDGF-BB (Jakob et al., 2001), especially when used in combination (TFP) (Barbero et al., 2004). Here we aimed at determining how kinetic parameters of AHAC growth are modulated by TFP. In particular, we first used microcolony assays (Sherley et al. 1995) to estimate the following kinetic parameters: (i) time of first cell division, (ii) cell division time of single cell (generation time, G_T), (iii) percentage of quiescent cells and (iv) fraction of cells that divide per generation time (F_c(T)). We then developed a mathematical model combining time-lag (delay) and logistic equations to capture the kinetic parameters and to enable the description of the complete growth process of the cell culture. Finally, using the experimental and mathematical methods, we assessed the growth kinetic parameters of AHAC from the same donor at different passages in culture, to determine whether prolonged expansion in the presence or absence of TFP induces an enrichment in the fraction of the fastest proliferating cells.

2 Material and methods

2.1 Cell culture

2.1.1 Cell isolation and expansion

Full-thickness human articular cartilage samples were collected from the femoral lateral condyle of three individuals (patient A: 52 years, patient B: 50 years, patient C: 52 years), with no history and no radiographic signs of joint disease, after informed

consent and in accordance with the local Ethical Commission. Human adult articular chondrocytes (AHAC) were isolated using 0.15% type II collagenase for 22 hours and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum, 4.5 mg/ml D-Glucose, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml L-glutamine (complete medium). The isolated AHAC were counted using trypan blue, plated in tissue culture flasks at a density of 10^4 cells/cm^2 and cultured in complete medium, either without growth factors (control medium, CTR) or with the addition of 1 ng/ml of Transforming Growth Factor-β1 (TGF-β1), 5 ng/ml of Fibroblast Growth Factor-2 (FGF-2) and 10 ng/ml of Platelet-Derived Growth Factor-BB (PDGF-BB) (growth factor medium, TFP) in a humidified 37°C/5% CO_2 incubator. When cells were approximately 80% confluent, first passage (P1) cells were rinsed with phosphate buffered saline, detached using 0.05% trypsin/0.53mM EDTA and frozen in complete medium containing 10% dimethylsulfoxid. AHAC were then used for the kinetic studies described below (i.e., growth curves and microcolony tests) either immediately after thawing (passage 1 cells, P1) or following an additional expansion for 2 weeks (passage 2 cells, P2).

2.1.2 Growth curves

AHAC were seeded in 6 well-plates in CTR or TFP medium at a density of 1000 cells/cm^2 and placed in a humidified 37°C/5% CO_2 incubator. Cell proliferation was assessed during 12 days' culture by repeated measures of cell numbers in the same wells (N=6 for each experiment) using alamarBlue^{TM} (a component transformed by living cells from the oxidized non-fluorescent state to the reduced fluorescent state). Briefly, at 12 hours intervals, culture medium was replaced with fresh medium containing 10% alamarBlue^{TM} solution (Serotec Ltd, Düsseldorf, Germany). After four hours, fluorescence intensity was measured (excitation: 560 nm; emission: 590 nm) and converted to cell numbers using a standard curve, generated in preliminary experiments by haemocytometer-based counting of trypsinized cells immediately after alamarBlue^{TM} assays. Morphological features of AHAC cultures in CTR and TFP media were monitored by phase contrast microscopy.

2.1.3 Microcolony tests

Cell culture flasks (150 cm^2) were prepared by drawing a grid below the culture surface (3mm-spaced horizontal and vertical lines). AHAC were seeded in the flasks in CTR or TFP medium at a density of 2.5 cells/cm^2. The use of such a low cell density allowed observation of microcolonies derived from single cells, whereas the use of the grid allowed to track the same microcolonies at different times. The number of cells in each microcolony was manually counted using phase contrast microscopy at 8 hour intervals for 6 days. The collected data were used to calculate: (i) the time of first cell division (t_{cd}), as the time (approximated as a multiple of 8 hours) required by each seeded cell to reach the stage of a 2-cell microcolony; (ii) the

generation time (g_T), as the time (approximated as a multiple of 8 hours) required by each 2-cell microcolony to reach the stage of a 4-cell microcolony (in preliminary experiments, we found that g_T was virtually identical to the time required by each 4-cell microcolony to reach the stage of a 8-cell microcolony; therefore, g_T can be considered as the cell division time following the first cell division); (iii) the percentage of quiescent cells (Q_c), defined as those which did not reach the stage of 2-cell microcolonies during the entire observation time; (iv) the fraction of cells that divide per generation period (f_c(T)), defined as

$$f_c(T) = \frac{\sum_{i=1}^{N-1} DF_i}{T/g_T}, \tag{2.1}$$

where DF_i (dividing fraction in the i-th interval) is the ratio between the new cells that appear in the i-th interval and the cells at the previous interval, N is the number of time intervals, and T is the total observation time (144 hours) (Sherley et al. 1995).

2.2 Mathematical model

2.2.1 Description of the model

An exponential model ($dN/dt = \rho N(t)$) assumes that all cells divide instantaneously, so that the growth rate at time t is proportional to $N(t)$, the number of cells at time t. Based on preliminary experiments we found that this assumption is not correct for AHAC, since the first cell division is not instantaneous. This prompted for the use of delay differential equations (DDEs) where the growth rate at time $t \geq G_T$ is set proportional to the cell number at some previous time $(t - G_T)$ (Baker et al., 1998), where G_T indicates the average generation time of the cell population.

We began with investigating the following simple delay model

$$\frac{dN}{dt} = \rho N(t - G_T), \quad t \geq 0 \tag{2.2a}$$

$$N(t) = \Psi(t), \quad -G_T \leq t < 0, \tag{2.2b}$$

where ρ is the cell proliferation rate and, assuming that there is no relevant cell death, corresponds to the number of offspring per parent cell in the population per time unit.

The experiment isassumed to start at time $t = 0$. Over the first interval [0, G_T), the rate of growth depends on some previous fictitious cell number. Hence it is necessary to specify a function $\Psi(t)$ over [-G_T, 0) that defines the rate at which new cells appear over [0, G_T). However, one should not interpret $\Psi(t)$ as the number of cells $N(t)$ for negative t in [-G_T, 0), but rather $\rho\Psi(t - G_T)$ as the rate of the cell growth for t in [0, G_T). If the growth is synchronous and the cells divide around

some specific time, $\Psi(t)$ should be a Gaussian centred about that time, but if the growth is asynchronous, $\Psi(t)$ should be a constant. In either case, the function is normalized by assuming that the number of cells duplicates over the first interval [0, G_T). Integrating (2.2a)-(2.2b) over [0, G_T), where $N(t - G_T) = \Psi(t - G_T)$ and imposing this normalization on $\Psi(t)$, we find the condition which the integral of $\Psi(t)$ must satisfy. Since our data presented an asynchronous behaviour we chose $\Psi(t)$ constant, with $\Psi(t) = N_0/(G_T\rho)$.

Beyond the seventh day the cell population encounters the physical limitation of the well size. To model the growth kinetics of the cells throughout the entire experiment, that is until confluence, we introduced a logistic delay equation

$$\frac{dN}{dt} = \rho N(t - G_T)\left(1 - \frac{N(t)}{K}\right), \quad t > 0, \quad N(0) = N_0 \tag{2.3a}$$

$$N(t) = \Psi(t), \quad -G_T \leq t < 0, \tag{2.3b}$$

Here the growth rate is no longer the single parameter ρ but is given by $\rho(1 - N(t)/K)$, which decreases to zero as the number of cells $N(t)$ tends to the constant K. The parameter K defines the "carrying capacity" of the environment. In our experiments, K is determined by the space available to the cells, i.e. the well area, since the medium supply is assumed abundant and constant. Hence the value of K corresponds to the maximal number of cells in the monolayer which can be reached at confluence; note that $N(t)$ tends to K as t goes to infinity for any initial value $N_0 > 0$. As the growth can be considered exponential during the first time interval [0, G_T), when N(t) is still very small, the normalization constraint on $\Psi(t)$ did not require a further adjustment.

Note, that equation (2.3a) has two steady states, $N = 0$ and $N = 1$. Perturbing (2.3a) about the state $N = 1$ one finds that this steady state is linearly stable. On the other hand, when we perturb the state $N = 0$, we find the linearized equation $dN/dt = N(t - G_T)$. Upon making the ansatz $N(t) = Cexp(-\rho\lambda G_T)$ we find that the solution to the transcendental equation $\lambda = exp(-\rho\lambda G_T)$ may have complex solutions in addition to one real positive solution. However, for values of G_T which are about 1, they all turn out to have negative real parts, and therefore the corresponding oscillatory solutions to equation (2.3a) are decaying. Hence, we expect the solution to have a non-oscillatory monotone increasing shape from $N = 0$ to the stable state $N = 1$ (Murray, 2002).

2.2.2 Numerical methods

The task of parameter estimation is one of minimising, in a least-squares sense, an objective function based on unknown parameters p and sample data $\{t_i, N_i = N(t_i)\}$, for t_i = 1,..., M. Given an initial value $N(t_0) = N_0$ and an initial function $\Psi(t)$ for t in [-G_T,0), each set of parameter values defines a solution N(t) = N(t; p) for $t \geq 0$, where $p = [\rho, G_T, K]$. We took as N_0 our first experimental data at time t = 0. To find the global best-fit parameter values p* to the data, the initial guess

must be sufficiently close to p*. The microcolony tests provided us with a good initial estimate for G_T. To compute N(t_i, p) the DDE is solved with an adaptive fourth-order Runge-Kutta method (Hainer et al., 1996). The nonlinear optimisation problem is solved by the Gauss-Newton method, combined with the Armijo rule for an optimal step length (Kelley, 2003).

2.3 Statistical analysis

Statistical evaluation was performed using SPSS software version 7.5 software (SPSS, Sigma Stat). Values are presented as mean ± standard deviation (SD). Differences between cultures in CTR and TFP medium of cells from the same donor were assessed by Student's t-tests for independent samples, after confirming the normality of the populations by skewness and kurtosis. Differences among donors were assessed by Mann Whitney tests for independent samples. P values less then 0.05 were considered to indicate statistically significant differences.

3 Results

3.1 Growth curves

Morphologically, CTR-expanded AHAC were flattened and spread, while TFP-expanded cells were generally smaller with a more elongated, spindle-like shape (Fig. 1A).

The growth curves of AHAC from all donors were typically sigmoidal (Fig. 1B): after a lag period of about 3-4 days, cells multiplied exponentially until day 9-10, when they reached the plateau phase. The density of cells counted in the presence of TFP medium was significantly higher than in CTR medium starting from day 5 and was 4.2-fold higher at day 12.

3.2 Microcolony tests

For each experiment, 20-60 microcolonies per flask were identified and the number of cells per colony was counted every 8 hours for a total time of 144 hours. The percentages of microcolonies containing 1, 2, or 4 cells were derived at each observation and used to generate microcolony profiles, as shown in Fig. 2. In CTR medium, the percentage of 1-cell microcolonies declined slowly, reaching a plateau of around 20% after approximately 100 hours, in parallel with a relatively slow increase in the percentage of 2-cell and 4-cell microcolonies. In the presence of TFP, the percentage of 1-cell microcolonies declined to 10% already after about 60 hours of culture, due to the rapid appearance of microcolonies with progressively increasing cell numbers (Fig. 2).

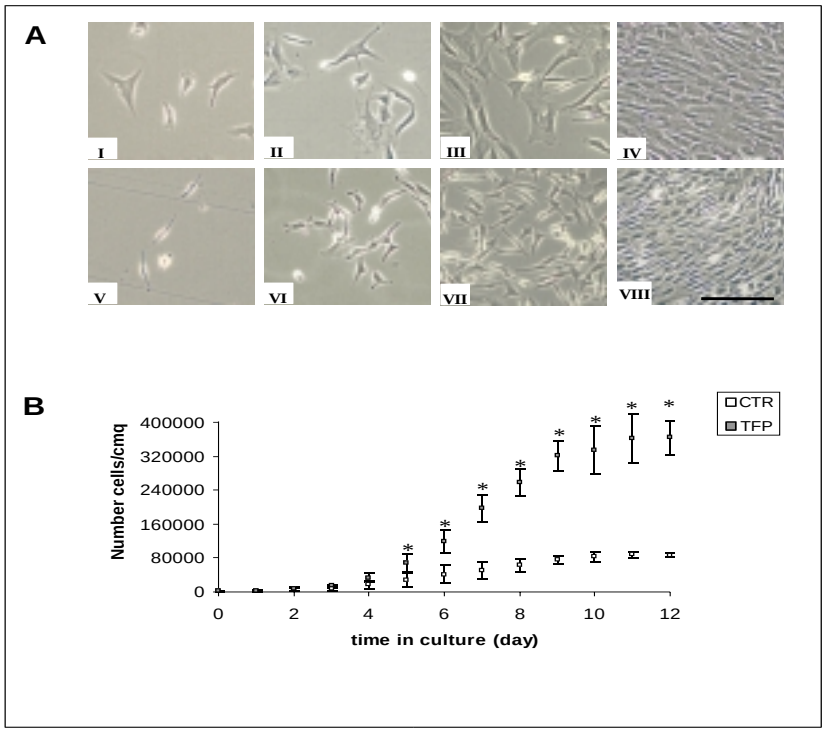

Fig.1

A: Representative pictures of adult human articular chondrocytes (AHAC) expanded for 1 (**I** and **V**), 4 (**II** and **VI**), 8 (**III** and **VII**), and 12 (**IV** and **VIII**) days in medium without growth factor, CTR (**I-IV**) or with the growth factor combination TGFβ1/FGF-2/PDGF-BB (TFP) (**V-VIII**). Bar = 100 μm.
B: Growth curves of AHAC expanded in CTR and TFP medium; cell number was measured fluorimetrically every 12 hours and normalized to the dish surface area; values are the mean $\pm$ SD of cells from 3 donors. * = P< 0.05 from AHAC expanded in CTR.

Remarkably, the percentage of microcolonies containing more than 4 cells was higher than 50% only at 115 ± 17 hours in CTR medium, but already at 80 ± 8 hours in TFP medium.

The collected data were then used to calculate the following kinetic parameters related to AHAC growth (Table 1):

Table 1. Microcolony growth parameters: differences between CTR- and TFP-expansion.

Summary of growth kinetic parameters estimated by microcolony tests. Parameters are reported as mean ± SD of microlonies within the same donor (§), or of mean values calculated for each donor ± SD (†). * = P < 0.05 from CTR medium.

	Expansion condition	donor A (mean ± SD§)	donor B (mean ± SD§)	donor C (mean ± SD§)	average (mean ± SD†)
Time of first cell division (hours)	CTR	48.6 ± 19.7	64.3 ± 22.6	53.6 ± 21.9	55.5 ± 8.0
	TFP	32.6 ± 15.5*	36.3 ± 11.3*	53.3 ± 16.9	40.7 ± 11.0*
Percentage of quiescent cells	CTR	19.0	21.2	30.3	23.5 ± 6.0
	TFP	7.7	11.8	21.2	13.5 ± 6.9*
Generation time ($g_T$hours)	CTR	29.2 ± 6.4	25.5 ± 10.4	31.1 ± 13.0	28.6 ± 2.9
	TFP	25.1 ± 11.1	20.6 ± 4.9*	25.6 ± 12.2*	23.8 ± 2.7*
Fraction of cell dividing per g_T	CTR	0.86 ± 0.08	0.87 ± 0.14	0.85 ± 0.14	0.86 ± 0.01
	TFP	0.85 ± 0.11	0.90 ± 0.15	0.87 ± 0.15	0.87 ± 0.02

(i) Time of first cell division. t_{cd} was highly variable (16-96 hours) even among cells from the same donor and cultured in the same medium, indicating large heterogeneity of different AHAC subpopulations. Despite these variations, the mean t_{cd} (T_{cd}) was significantly shorter (1.4-fold, corresponding to 14.8 hours) if cells were cultured in TFP, as compared to CTR medium.

In order to further quantify differences between CTR- and TFP-expanded AHAC, microcolonies were arbitrarily divided into the following three groups: group I for $t_{cd} \leq 16$ hours, group II for t_{cd} between 17 and 32 hours, and group III for $t_{cd} \geq 33$ hours. As compared to CTR-expanded AHAC, those expanded in TFP contained a statistically significant higher fraction of microcolonies in group I (0.7% vs 9.1%) (Fig. 3A).

(ii) Percentage of quiescent cells. Q_c was 1.7-fold lower in AHAC cultivated in the presence of TFP as compared to CTR medium, indicating that the growth factor mix induced a significant increase in the proportion of mitotically active cells.

(iii) Generation time. g_T was highly variable (12-72 hours) even among microcolonies from the same donor and cultured in the same medium, again underlining a large heterogeneity of different AHAC subpopulations. Despite these variations, the mean g_T (G_T) was significantly shorter (1.2-fold, corresponding to 4.8 hours) in AHAC cultivated in the presence of TFP as compared to CTR medium. Microcolonies were again classified into the following three groups: groupI for $g_T \leq 16$ hours, group II for g_T between 17 and 32 hours, and group III for $g_T \geq 33$ hours. As compared to CTR-expanded AHAC, those expanded in TFP contained a statistically significant higher fraction of microcolonies in group I (11.9% vs 25.4%) and a lower fraction of microcolonies in group III (23.4% vs 11.7%) (Fig. 3B).

(iv) Fraction of cells that divide per g_T. $f_c(T)$ is an index of the "exponentiality" of the cell growth, calculated for each microcolony ($f_c(T)$ equals 1 for an ideal exponential growth, and 0.5 for a linear population growth). The mean $f_c(T)$, $F_c(T)$, was similar in CTR- and TFP-expanded populations and close to 1, indicating a common exponential pattern of cell growth.

Table 2. Growth parameters calculated by the exponential delay model: differences between CTR- and TFP-expansion.

Values of G_T and ρ are fitted by the delay model (2.2a)–(2.2b) to the experimental data, values of D_T are calculated using the following formula: $D_T = \ln(2)/(\rho\lambda)$. All values of G_T and D_T are given in hours.
In the last column, mean values calculated from each donor ± SD are reported.

	donor A			donor B			donor C			average		
	G_T	ρ	D_T	G_T	ρ	D_T	G_T	ρ	D_T	G_T (mean ±SD)	ρ (mean ±SD)	D_T (mean ±SD)
CTR	27.0	0.86	33.7	19.5	0.75	33.3	29.8	1.10	30.1	25.4 ± 5.3	0.90 ± 0.18	32.4 ± 2.0
TFP	24.0	1.60	22.1	17.0	1.10	24.5	28.0	1.40	25.4	23.0 ± 5.6	1.37 ± 0.25	24.0 ± 1.7

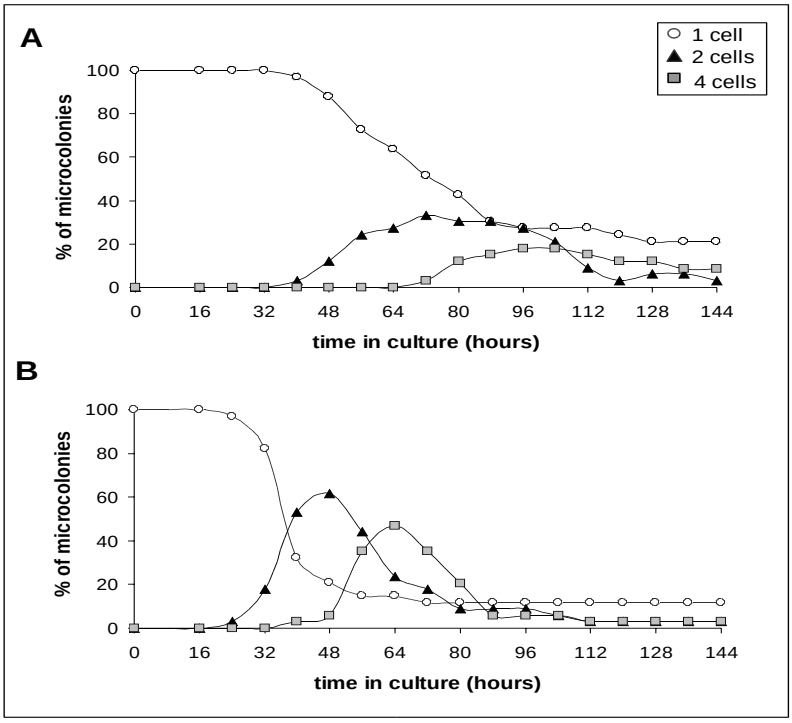

Fig.2

Microcolony progression analysis of AHAC from one characteristic donor (donor A) expanded in CTR (**A**) or TFP (**B**) medium. Cells were plated at a low density in culture flasks as described in *Material and Methods*. Every 8 hours, number of cells per microcolony were counted under phase contrast microscopy. The number of a given microcolony type observed is expressed as a percent of the total observed. Microcolonies with three cells were omitted for simplicity.

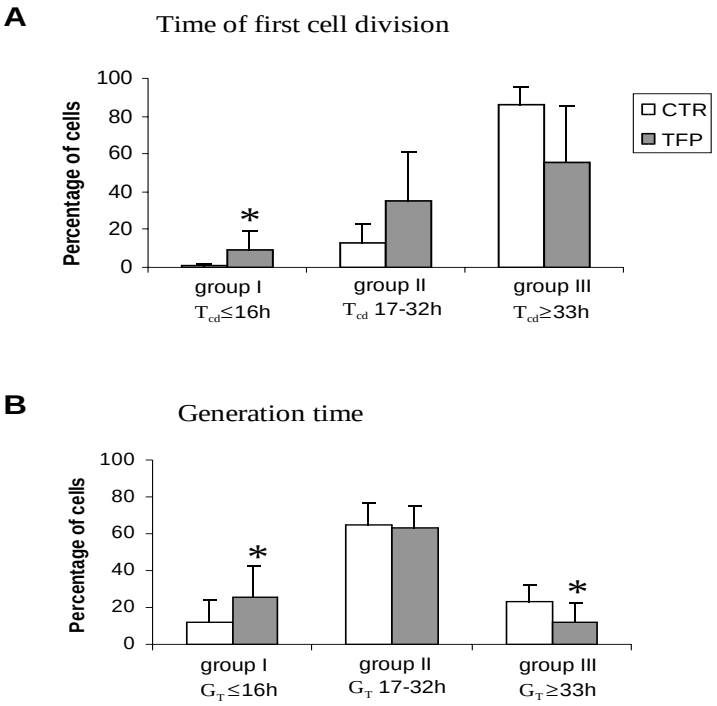

Fig.3

Time of first cell division (**A**) and generation time (**B**) of cells expanded in CTR or TFP medium, following subdivision in arbitrary groups. * = P< 0.05 from AHAC expanded in CTR.

3.3 Mathematical model

To determine G_T and ρ, the delay model (2.2a)-(2.2b) was first fitted to the measurements from the three experiments during the exponential phase only. The resulting values for G_T were similar to those obtained from the microcolony test (Table 1 and 2). Between the two expansion conditions (in CTR or TFP medium), only a slight difference in G_T was observed (average variation = 9.4%) , while in general there was a large difference in ρ (average variation = 34.3%) (Tab.2). This can be explained by the fact that ρ represents the overall proliferation rate taking into account the number of quiescent cells and the time of first cell division, parameters that have been shown to have higher values in CTR medium.

In the study of cell growth dynamics, another typical parameter is the doubling time D_T. For a simple exponential model (without delay), D_T is constant and equal to $\ln(2)/\rho$. However, in our exponential model with delay, the doubling time varies with time. It nevertheless reaches an asymptotic limit (D_T) at long times, as transients during initial stage are dissipated. Clearly, the asymptotic value D_T depends on G_T and ρ. As the behaviour of the delay model tends to that of an exponential model at long times, we can seek for a solution of (2.2a)-(2.2b) of the form

$$N(t) = C \exp(\rho\lambda t)\,, \tag{3.1}$$

where C is some positive constant. We introduce (3.1) into (**??**) which leads to the transcendental equation

$$\lambda = \exp(-\rho\lambda G_T)\,, \tag{3.2}$$

By solving this equation for λ with Newton's method for different values of G_T and ρ, we then calculate $D_T = \ln(2)/(\rho\lambda)$. In Fig. 5 the relationship between D_T and G_T for a typical value of ρ is shown. We observed that D_T increased with increasing G_T in a nonlinear way (Fig. 4). In Table 2 the values of D_T, extrapolated for each donor and expansion condition, are reported. Interestingly, while G_T varied substantially between different donors, D_T remained almost similar in all experiments (% variation: about 20% for G_T and 7% for D_T).

Table 3. Carrying capacity (K) calculated by the logistic delay model: differences between CTR- and TFP-expansion.

Values of K are fitted by the delay model (2.3a)–(2.3b) to the experimental data. In the last column, mean values calculated from each donor ± SD are reported.

	donor A	donor B	donor C	average (mean ± SD)
CTR	10.1	12.6	8.7	10.5 ± 2.0
TFP	40.4	37	38.0	38.5 ± 1.8

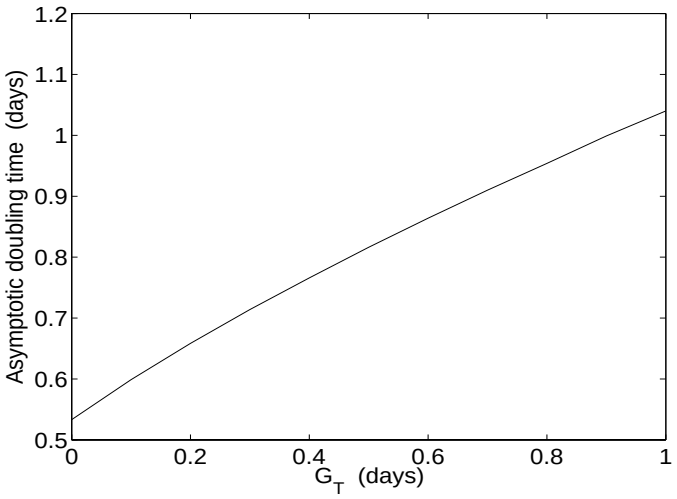

Fig. 4. Relationship between generation time (G_T) and doubling time (D_T). Values of these parameters plotted in this diagram were obtained from cells derived from the donor B and expanded in TFP ($\rho = 1.1$).

Since the fit to the exponential delay model yielded a good estimate of G_T, as confirmed by the microcolony tests, these values were then used in the logistic delay model to obtain the carrying capacity, K. Consistently with the experimental data, K was four times larger in the presence of TFP than in CTR medium, probably due to the efficiency of space occupation (Table 3). A reduction of the error between a standard logistic model and our delay logistic model (2.3a-2.3b) was observed (the mean error in the former was 1.17 and 1.06 respectively for CTR and TFP medium, but only 0.69 and 0.96, respectively, in the second); hence the new model better approximates the observations. In Fig. 5 we show the solutions obtained by fitting the delay logistic model (2.3a-2.3b) to the experimental data for each donor in the two expansion conditions.

Table 4. Microcolony growth parameters: differences between first (P1) and second passage (P2). Summary of growth kinetic parameters derived for cells from patient C cultured for one passage (P1) or two passages (P2) in CTR or TFP medium.
* = P < 0.05 from P1 cells.

	CTR		TFP	
	P1	P2	P1	P2
Time of first cell division (hours)	53.6 ± 21.9	41.9 ± 18.3*	53.3 ± 16.9	25.5 ± 14.7*
Percentage of quiescent cells	30.3	4.4	21.2	2.6
Generation time (g_T, hours)	31.1 ± 13.0	27.5 ± 9.7	25.6 ± 12.2	26.1 ± 7.9
Fraction of cells dividing per g_T	0.85 ± 0.14	0.88 ± 0.13	0.87 ± 0.1	0.85 ± 0.12

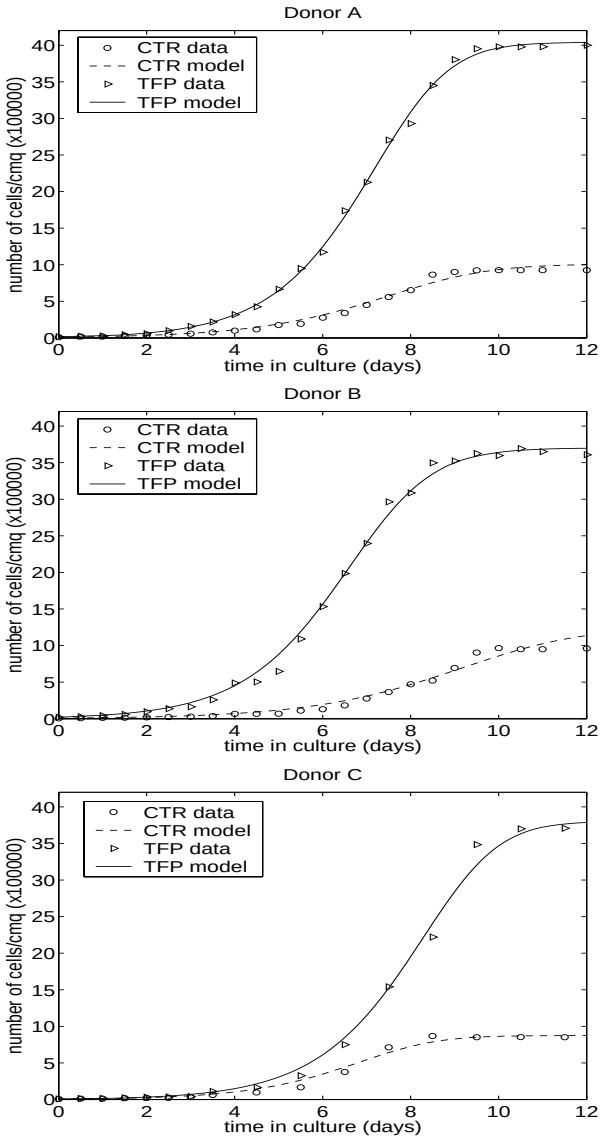

Fig. 5. Experimental ($\triangle$ for CTR and O for TFP medium) and predicted (- - - for CTR, — for TFP medium) growth curves of cells from donors A, B or C.

3.4 Differences in the growth kinetics between AHAC at different passages

An intriguing question is whether prolonged expansion in the presence or absence of TFP could induce an enrichment of AHAC populations with the fastest growth capacity. To address this question, AHAC from one donor (donor C) were expanded in CTR or TFP medium either for one (P1 cells) or two (P2 cells) passages, corresponding respectively to 1.4 or 13.3 doublings for CTR and 2.5 or 17.2 doublings for TFP, and then assessed using the microcolony test in combination with the developed mathematical model. Unexpectedly, no difference was observed in the G_T measured using P1 or P2 cells expanded in CTR or TFP medium (Table 4), and the percentage of fast subpopulations (group I) in P2 cells was lower than in P1 cells (2.2 and 4.4-fold respectively for CTR and TFP) (Fig. 6 A and B). The accuracy of the experimentally determined G_T was confirmed by the fact that the mathematical model was able to predict the effective temporal growth in cell number only if the measured G_T, but not a shorter G_T, was given as input (Fig. 7). Interestingly, as compared to P1 cells, P2 cells had a shorter T_{cd} (1.3-fold, corresponding to 11.7 hours, in CTR medium and 2.1-fold, corresponding to 27.8 hours, in TFP medium) and a lower Q_c (6.9-fold and 8.2-fold respectively for CTR and TFP medium) (Table 4). Moreover, prolonged expansion induced an enrichment of cells with short T_{cd} (groups I and II) especially using TFP medium (Fig. 6 B,C).

4 Discussion

In the present study, we used a combination of microcolony tests and a newly developed mathematical model, combining logistic growth with time delay, to (i) measure the kinetic parameters of AHAC, (ii) capture the entire growth process, and (iii) investigate the specific effects of the growth factor combination TFP on cell proliferation. We found that TFP medium increases the number of chondrocytes in monolayer culture by (i) increasing the percentage of quiescent cells (Qc), (ii) reducing the mean time required for single cells to enter the first division (Tcd), and (iii) reducing the mean cell division time of cells (generation time, G_T). Our mathematical model confirmed the value of G_T and provided estimates for the carrying capacity of the system (K) and the proliferation rate (ρ), which were respectively 3.7- and 1.5-fold higher in chondrocytes cultured in TFP. Moreover, our results for chondrocytes from the same donor at different passages in culture indicate that prolonged expansion does not increase the fraction of the fastest proliferating AHAC, but rather the fraction of AHAC with a higher propensity to initiate duplication, particularly in the presence of TFP.

Sherley et al. (Sherley et al., 1995) first used the microcolony test as a tool to investigate changes in growth properties of mouse mammary epithelial cell lines under condition inducing elevated p53 expression. We also adopted the microcolony test to study the growth kinetic of AHAC and its modulation due to the presence

of TFP medium. Ascompared to CTR-expanded AHAC, those expanded in TFP contained ahigher fraction of cells with short generation time (less than 16 hours) and a lower fraction of AHAC with high generation time (more than 32 hours). However, based on our experimental data, we could not determine whether all the cells in culture or only specific cell subpopulations responded to TFP by reducing their g_T. Interestingly, the overall growth pattern of chondrocytes was not altered by TFP, as indicated by the fact that identical values of the index of the exponential nature ($F_c(T)$) were obtained in chondrocytes cultured with and without growth factors. The finding that $F_c(T)$ was close to 1 in both conditions allowed us to use an exponential model for the initial phase of cell growth. One striking observation was that the time of first division and the generation time greatly differed among microcolonies derived from different cells of the same patient, in agreement with a previously described large heterogeneity of different AHAC clonal subpopulations (Barbero et al., 2003).

To capture the non-instantaneous and asynchronous first cell division, a delay model had to be adopted. Using a delay model it is in fact possible to distinguish between G_T, a characteristic of a single cell, and the doubling time (D_T), a global feature of the whole cell population. G_T is clearly shorter than the doubling time, since it does not take into account the quiescent cells and the delay in the first cell division. We observed that $D_T$always tends to an asymptotic value, different for different values of G_T, and more interestingly, that there is a relationships between G_T and the asymptotic value of D_T; this relationship depends on the proliferation rate (ρ), but not on the initial cell number. Once having estimated G_T and ρ by fitting our model to the data, we can thus extrapolate the correspondent value of D_T from the graph.

Our model characterised cell growth during the entire experiment, until confluence. This allowed us to estimate the number of cells at confluence (K), which was remarkably different between the two expansion conditions (CTR or TFP medium). This difference can be explained by the smaller and more elongated cell shape induced by TFP. Further studies have to be performed to assess whether cells cultured in TFP medium have also an increased tendency to migrate, which would lead to a more efficient occupation of the available space.

In this study we also aimed at determining whether prolonged expansion in the presence or absence of TFPinduces an enrichment of the populations with the highest growth capacity. Unexpectedly, we observed that P1 and P2 chondrocytes divided with unchanged mean G_T, and, more interestingly, that the fraction of fast cells (G_T lower than 16) decreased dramatically from P1 to P2 chondrocytes. These differences were more pronounced if cells were expanded in TFP medium. Since replicative aging occurs during *in vitro* cell expansion and the senescence-mediated phenomena become more evident in cells undergoing elevated population doublings (Harley et al., 1990), it is possible to speculate that cell senescence following prolonged expansion masked a possible selection of the fast chondrocytes.

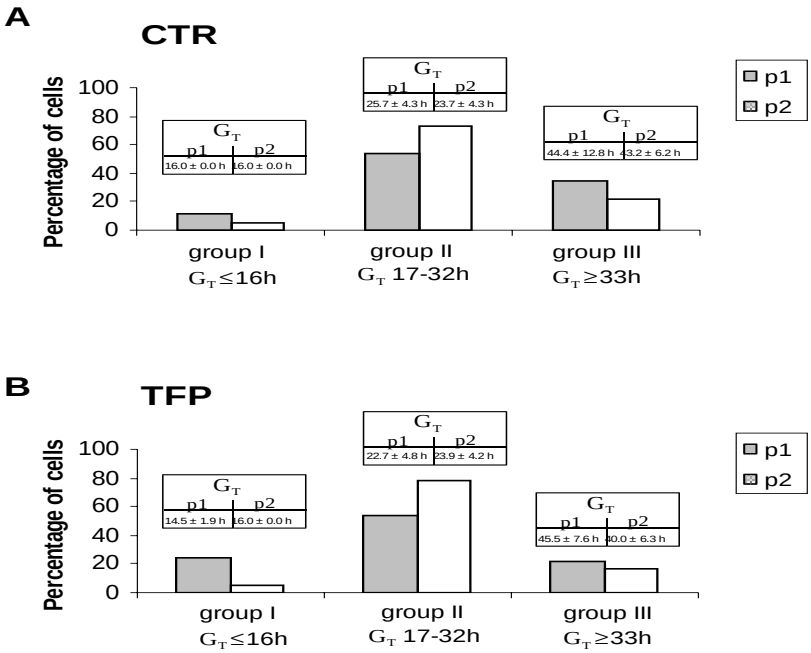

Fig.6

Generation time (G_T) (**A** and **B**) and time of first cell division (T_{cd}) (**C** and **D**) in P1 and P2 cells expanded in CTR (**A** and **C**) or TFP (**B** and **D**) medium, following subdivision in arbitrary groups. * = P< 0.05 from P2 cells.

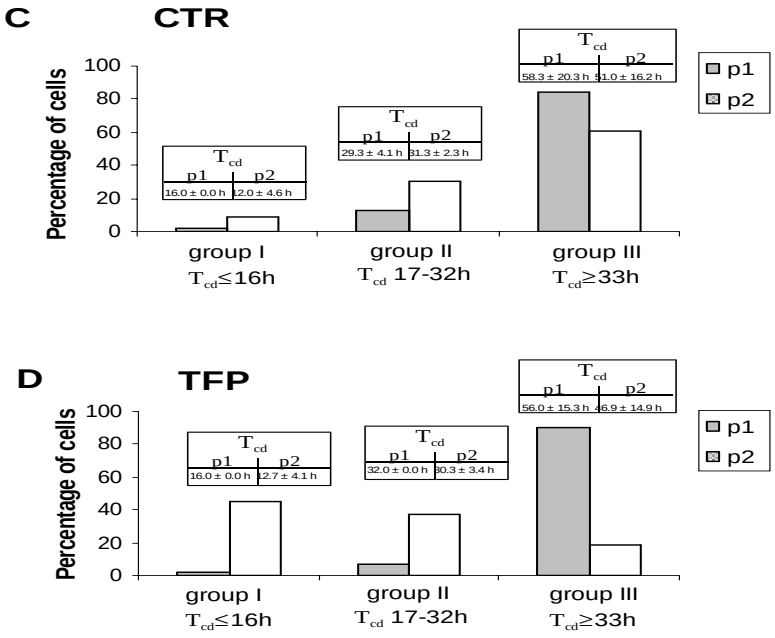

Fig.6 continuum

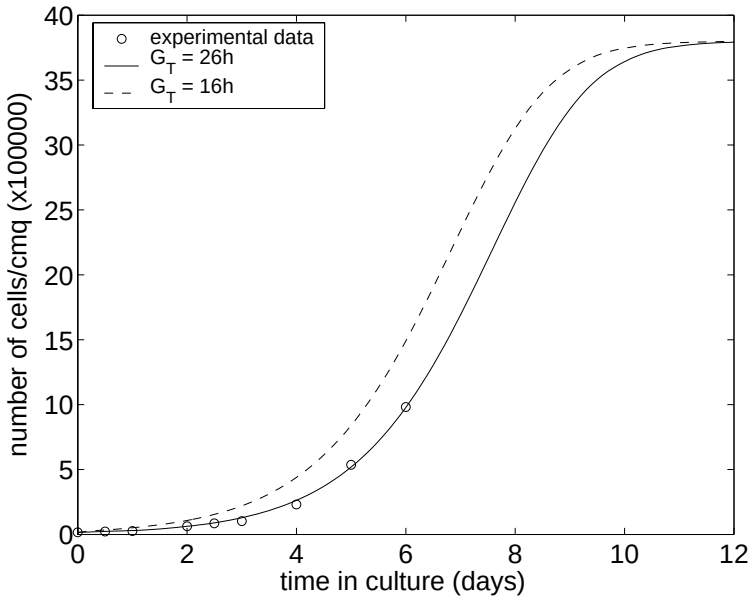

Fig. 7. Growth curves of P2 cells from donor C obtained applying G_T of 16 (- - -) or 26 (—) hours in the logistic model. Describe that the GT of 16 did not fit. The circles (°) indicate the experimentally determined number of cells.

On the other hand, P2 chondrocyte population had shorter T_{cd}, larger fraction of cell with short t_{cd}, and lower Q_c than P1 cell population, especially if cells were expanded in TFP medium. This result indicates that prolonged expansion, particularly in medium containing TFP, might induce a selection of chondrocytes with a higher propensity to initiate duplication.
In the present work, we have studied the influence of the growth factor combination TGFβ1, FGF-2, and PDGF-BB on the growth kinetic of adult human articular chondrocytes using both microcolony tests and a mathematical model. The described approach could be adopted to quantitatively assess the growth of other cell types, cultured under different experimental conditions.

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