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Real-Time Measurements of Human Chondrocyte Heat Production During In Vitro Proliferation

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ABSTRACT: Isothermal microcalorimeters (IMC) are highly sensitive instruments that allow the measurement of heat flow in the microwatt range. Due to their detection of minute thermal heat, IMC techniques have been used in numerous biological applications, including the study of fermentation processes, pharmaceutical development, and cell metabolism. In this work, with the ultimate goal of establishing a rapid and real-time method to predict the proliferative capacity of human articular chondrocytes (HAC), we explored to use of IMC to characterize one of the crucial steps within the process of cartilage tissue engineering, namely the in vitro expansion of HAC. We first established an IMC-based model for the real-time monitoring of heat flow generated by HAC during proliferation. Profiles of the heat and heat flow curves obtained were consistent with those previously shown for other cell types. The average heat flow per HAC was determined to be 22.0 ± 5.3 pW. We next demonstrated that HAC proliferation within the IMC-based model was similar to proliferation under standard culture conditions, verifying its relevance for simulating the typical cell culture application. HAC growth and HAC heat over time appeared correlated for cells derived from particular donors. However, based on the results from 12 independent donors, no predictive correlation could be established between the growth rate and the heat increase rate of HAC. This was likely due to variability in the biological function of HAC derived from different donors, combined with the complexity of tightly couple metabolic processes beyond proliferation. In conclusion, IMC appears to be a promising technique to characterize cell proliferation. However, studies with more reproducible cell sources (e.g., cell lines) could be used before adding the complexity associated with primary human cells.

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KEYWORDS: calorimetry; tissue engineering; cartilage; metabolism

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Introduction

Isothermal microcalorimeters (IMC), which have the capacity to measure heat flow in the range of microwatts, have long been used as analytical tools for thermodynamic and kinetic studies (Wadsö, 1996, 2002). In addition to the high thermal sensitivity, the power of IMC techniques is also related to the ability to perform rapid, passive, non-invasive, and real-time measurements (Braissant et al., 2010; Lewis and Daniels, 2003). Moreover, during heat flow data acquisition, samples can be kept in sealed ampoules, intact for further investigations, thus allowing IMC to be used as a non-destructive technique.

Beyond the more traditional thermodynamic applications within the physical sciences, a sufficiently sensitive calorimeter can be used in the study of living systems, for instance to quantify cell metabolic activities. Calorimetry-based techniques have been employed in a broad range of biological applications including fermentation (Wadsö and Gomez Galindo, 2009), pharmaceuticals (Buckton, 1995; Tan and Lu, 1999), and environmental studies (Rong et al., 2007). The use of IMC as a fast and inexpensive alternative to traditional diagnostic and prognostic tools has also been investigated for clinical applications (Monti, 1990), for example, in the rapid detection of bacterial infections (von Ah et al., 2009; Xi et al., 2002; Yang et al., 2008) and to monitor the metabolic activity of tumor cells (Bäckman, 1990).

Despite the power and sensitivity of calorimetric techniques for biological applications, such methods have

yet to be implemented within the rapidly growing field of cellular therapy and tissue engineering. The generation of cellular tissue grafts in vitro provides an attractive alternative to traditional treatments aimed at repairing or replacing damaged tissues to establish normal function. Calorimetry has the potential to represent an innovative tool to define and optimize specific cell culture parameters, as well as to provide non-invasive and non-destructive methods in quality control assessments (Kemp and Guan, 2000).

Currently, many clinically available cell therapy products for the repair of cartilage lesions involve a process of in vitro cell expansion. In this process, a limited number of chondrocytes, which can be obtained from the digestion of a small cartilage biopsy (only 100–250 mg of tissue), must be extensively proliferated on plastic dishes in order to obtain a sufficiently high number of cells to be re-implanted back into the patient. This step of the manufacturing process is lengthy, labor-intensive, and challenging to standardize. Establishing physics-based model systems aimed at better understanding and predicting chondrocyte function during proliferation could help in the development of protocols to reduce the time of expansion and improve its reproducibility, ultimately having a great potential impact on the manufacturing process.

Therefore, in this study, we aimed to first establish an experimental setup for the real-time monitoring of heat flow generated by human articular chondrocytes (HAC) during their in vitro proliferation. We next aimed to assess whether HAC proliferation within the IMC-based model system was similar to proliferation under well-established conventional culture conditions, verifying its relevance for predicting results of traditional cell and tissue culture applications. Finally, with the goal of establishing a rapid and real-time calorimetric method to predict the proliferative capacity of HAC under defined in vitro culture conditions, we assessed whether there was a correlation between the growth rate of HAC during the exponential phase of proliferation and the heat increase rate determined by the IMC-based method.

Materials and Methods

Cell Isolation and Expansion

Human articular chondrocytes were isolated from cartilage biopsies obtained post-mortem from 12 individuals (ages 65 ± 13 years) after informed consent and in accordance with the local Ethical Commission. As previously described (Jakob et al., 2001), HAC were isolated using 0.15% type II collagenase (10 mL solution/g tissue) for 22 h and resuspended in high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen #10938-025, Basel, Switzerland) supplemented with 10% fetal bovine serum, 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 chondrocytes were expanded for 1 passage (4–5 doublings) in culture medium supplemented with 1 ng/mL of transforming growth factor-β_1 (TGF β_1), 5 ng/mL of fibroblast growth factor-2 (FGF2) and 10 ng/mL of platelet derived growth factor-BB (PDGF-BB), a cocktail of factors previously shown to increase human chondrocyte proliferation (Jakob et al., 2001). Twelve hours prior to calorimetry experiments, HAC were synchronized with Aphidicolin (1 μg) (Saris et al., 1999); this compound does not affect cell viability or "S" phase duration, does not interfere with the synthesis of dNTPs or DNA polymerases, thus permitting to obtain a population of cells ready to start the replication when the block is removed. Synchronized HAC were then trypsinized, and either replated and cultured under conventional cell expansion conditions (i.e., in 6 wells plates, 10,000 cells/well with 2 mL medium, within a 37°C 5% CO_2 incubator), or seeded in calorimetry ampoules (100,000 cells/ampoule in 3 mL medium). Culture medium in the wells was exchanged twice weekly, whereas the microcalorimetry ampules were sealed, and thus no culture medium or gas exchange could be performed throughout the culture period.

Heat Flow Measurement

A multi-channel IMC (TAM III, TA Instruments, New Castle, DE) equipped with 48 measuring channels was used to monitor the heat production by HAC. The IMC instrument was thermostated to 37°C, 2 days before the start of experiments to achieve maximum stability. The heat flow signal was acquired continuously and resampled to obtain an effective sampling rate of one data point every 300 s, for up to 6 days. Glass ampoules having a total volume of 4 mL, loaded with 3 mL of cell suspension, were introduced into the TAM III channels, using a two step temperature equilibration procedure: the samples were first lowered into an equilibration position, and only after 15 min they were further lowered into the measuring position (von Ah et al., 2009). Data acquisition started 45 min after samples were lowered in the measurement position. Ampoules containing only cell-free culture medium were placed in three channels as controls, in order to assess background heat production resulting from the potential degradation of medium components. All samples were assayed in duplicate or triplicate.

Cell Quantification

The cell numbers were assessed every 24 h (in the first experiment every 12 h) in the calorimetry ampoules and every 48 h in the 6-well plates. Both microcalorimetry ampoules and wells were rinsed with PBS (Gibco, Grand Island, NY), frozen (−20°C) and then washed in 300 μL of 1% SDS to collect the DNA. Aliquots of this solution were analyzed using the CyQUANT Cell Proliferation Assay Kit

(Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Moreover, at the time of each seeding, three aliquots of the seeded HAC cell suspension were collected. Cells were centrifuged to obtain pellets, which, after freezing, were digested with 1% SDS. Since these samples contained a known number of cells they served as a reference to correlate the DNA content with the cell number.

Cell Growth Rate Determination

The growth of HAC, in a defined time window, can be described by means of an exponential law (Barbero et al., 2005)

$$N(t) = N_0 e^{\mu_G t} \quad (1)$$

where $N(t)$ is the number of cells at time t and μ_G is the growth rate of HAC (cell doublings per day). We hypothesized that the growth rate is the same for HAC cultured in the ampoules and in the wells. As a consequence, the experimental data was fit by the use of a model described by three independent parameters: N_{0W}—the initial number of cells in the wells, N_{0A}—the initial number of cells in the ampoules, and μ_G. The Levenberg–Marquardt algorithm from the proFit code 6.2.0 (Quantum Soft, Uetikon am See, Switzerland) served for combined fitting of the two sets of experimental data and for deriving the related error of the three independent parameters. In order to obtain a reasonable estimate of the errors of the measures to be included into the fit, the following procedure was carried out. For both tubes and wells, three specimens were harvested every 24 ± 1 h to determine the cell number via DNA quantification. For each individual experiment the average of the cell number including standard deviation was examined. Subsequently, the coefficient of variation CV, defined as ratio between standard deviation and average value, was calculated. The mean CVs averaged through all days and donors for both data sets were used to estimate the error in cell number quantification.

Heat Increase Rate Quantification

The heat generated over time, $Q(t)$, by a number of various cell types has previously been shown to fit an exponential equation (Kimura and Takahashi, 1985)

$$Q(t) = Q_0 e^{\mu_Q t} \quad (2)$$

where μ_Q will here within be defined as the "heat increase rate." Given that Q is the integration of the heat flow Φ [Watts], which is measured directly by the microcalorimeter, then Equation (2) becomes

$$\Phi(t) = \frac{dQ(t)}{dt} = Q_0 \mu_Q e^{\mu_Q t} \quad (3)$$

This model was applied for $t_{equil} < t < t_{max}$ where t_{equil} was the time needed for the full equilibration (10 h), and t_{max} was the time at which the maximum heat flow was reached. The error of $\Phi(t)$ was 0.2 μW, according to the manufacturer of the microcalorimeter and the error of the measurement time was 2 s. Again, the Levenberg–Marquardt algorithm from the proFit code 6.2.0 was applied to fit the experimental data in order to determine μ_Q.

Results and Discussion

First, we developed an experimental setup to assess the feasibility to apply IMC for real-time monitoring of the heat flow of HAC. As controls, ampoules filled only with culture medium (cell-free) were assessed in the IMC in order to quantify the potential heat flow resulting from degradation of medium components. Measurements of the control samples remained constant at background levels throughout the culture period, indicating that the culture medium per se would have a negligible contribution to the overall heat flow in cell-based experiments. Since no data are currently available on the typical heat flow per cell of HAC in culture, we next aimed to optimize the number of cells in relation to the IMC heat flow measurement sensitivity. When 5E + 04 HAC per ampoule were assessed, a significant lag time was observed before growth related heat flow was sufficient such that measurements could be acquired above instrument background levels. When 5E + 05 HAC were assessed, the heat flow curve reached a plateau rapidly, that is, in several hours, therefore not allowing for the calculation of dynamic parameters. Alternatively, when 1E + 05 HAC were seeded per ampoule, the heat flow quickly rose above the instrument detection limit (baseline), then steadily increased within the first day, reached a maximum between 3 and 4 days, and then gradually decreased. Heat flow curves, shown in Figure 1a, were consistent in shape to those typically reported in the literature for other cell types (Karnebogen et al., 1993; Ma et al., 2007; Murigande et al., 2009; Nedergaard et al., 1977). Integrating the heat flow data over time, sigmoidal heat curves were generated (Fig. 1b), which were also consistent with those previously reported for other cell types (reviewed in Braissant et al., 2010). Therefore, in all subsequent experiments, 1E + 05 HAC were seeded into the ampoules.

Quantifying the number of adherent cells within the ampoules at different time points of culture, we observed that cells reached confluence at day 3, with the number of HAC plateauing at $4.1E + 05 \pm 8E + 04$. Interestingly, the maximum peak in the heat flow curve (8.9 ± 2.3 μW) also corresponded to the time point of 3 days. Therefore, at

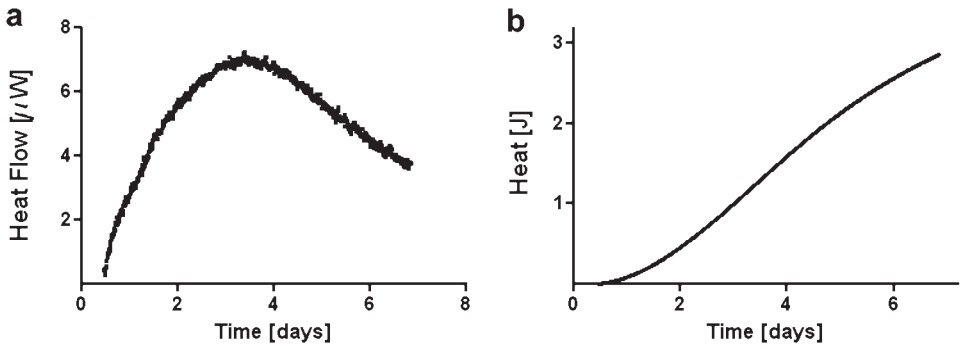

Figure 1. **a**: Real-time monitoring of the heat flow of HAC during in vitro proliferation when an initial cell density of 100,000 cells/ampoule was used. **b**: Heat curves generated by integrating the heat flow data over time. The peak in the heat flow curve, corresponding to the inflection point of the sigmoidal heat curve, occurred in the same time period as when HAC reached confluence in the IMC ampoules.

confluence, the heat flow per HAC was calculated to be 22.0 ± 5.3 pW. This value is in the range of those previously reported for fibroblasts (17 pW per mouse fibroblast, 40 pW per human fibroblast) (Kemp, 1991), which have a size and phenotype resembling in vitro expanded (i.e., dedifferentiated) HAC. Next, given that the heat curve has been directly related to biomass production for several cell types (Barros et al., 2003; Karnebogen et al., 1993; Sharma and Jain, 1990), we compared HAC growth up to confluence with the heat curve up to the inflection point (corresponding to the maximum point of the heat flow curve). Figure 2 shows that HAC heat production and HAC growth appeared to be related, and within this time frame seemed to follow exponential trends.

Based on the relation shown in Figure 2, we next aimed to determine whether there was a quantitative correlation between the heat increase rate and the growth rate during the exponential phase of HAC proliferation. This would allow establishing a rapid and real-time method to predict the proliferative capacity of HAC under defined in vitro culture conditions. However, for the IMC-based method to be relevant for cell and tissue culture applications, HAC proliferation within the ampoule/IMC-based model system must reflect proliferation under well-established conventional culture conditions. This needed to be validated given the different substrate materials (plastic vs. glass) and considering the sealed ampoules do not allow the same gas exchange as well plates. Figure 3 shows that the number of cells quantified in ampoules and in standard multi-well culture plates grow exponentially over time. Although both

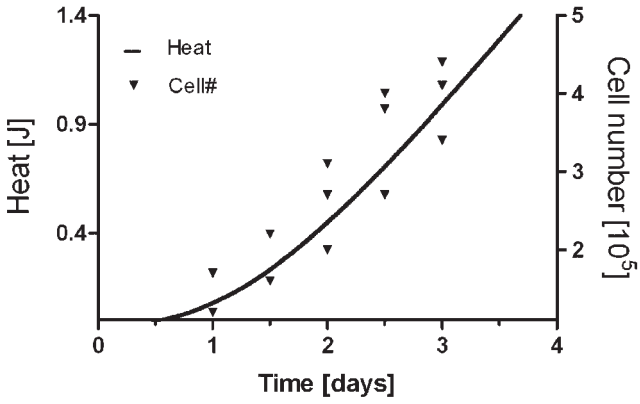

Figure 2. Relationship between heat and cell growth. Triangles in the graph represent the number of cells quantified in ampoules every 12 h, and the solid line represents the heat curve. For this donor, the growth of HAC and the heat both appear exponential and to be strongly related.

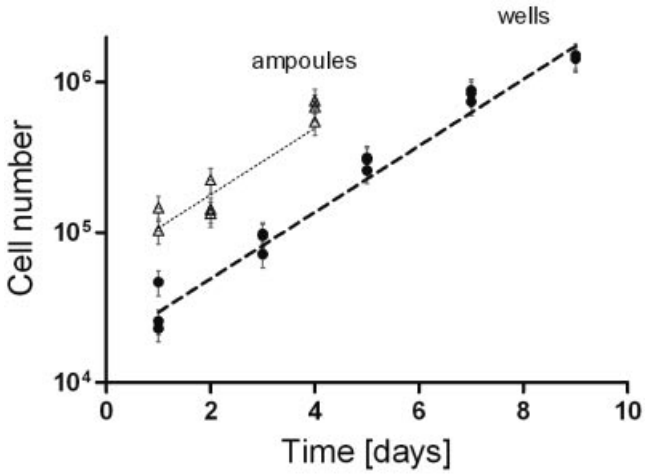

Figure 3. HAC growth within the ampoule/IMC-based model system and under conventional culture conditions in standard multi-well plates. Due to different surface areas available for the adherent cells the initial and final numbers of cells were different in the ampoules and in the well plates, as well as the time to reach confluence. However, the growth rates (i.e., slope of logarithmic plot) appeared similar between the two model systems. (Error bars for cell number are smaller than data points.)

the initial and final number of cells are different in the ampoules and in the well plates, due to the different surface areas available for the adherent cells, Figure 3 shows that the growth rates (i.e., slope in logarithmic plot) of HAC were similar within the two systems. Growth rates of HAC derived from different donors were variable, in the range of 0.2–0.9 doublings/day, consistent with rates previously found for HAC (Jakob et al., 2001). Therefore, sealed IMC ampoules provide a relevant environment and offer the potential to use resultant heat flow data for fast, online, and high-throughput monitoring of chondrocyte growth.

The growth rates (μ_G) of HAC derived from 12 different donors were plotted against the corresponding heat increase rates (μ_Q) in Figure 4. Regression analysis indicated a very weak linear correlation between the two parameters ($r^2 = 0.15$, $P = 0.00063$). As can be seen in Figure 4, the values of μ_Q were larger than the corresponding values of μ_G, highlighting that the rate of heat generated is function of not only the metabolic activities associated with cell proliferation, but of other ongoing biological processes as well.

In its native milieu, HAC have a very slow growth rate since the primary function of HAC in vivo is the gradual degradation and production of extracellular matrix proteins that comprise the cartilage tissue. For tissue engineering applications, the limited number of HAC that can obtained from a small cartilage biopsy must be induced to extensively proliferate in vitro in order to obtain a sufficiently high number of cells, which can then be implanted back into the patient. However, in addition to simply replicating, proliferating HAC may also synthesize ECM macromolecules such as collagen type I and versican (Jakob et al., 2001), which would contribute to overall metabolic heat production by the cells. Interestingly, the specific types of ECM proteins that are produced by HAC are known to be modulated by the stage of differentiation of the cell (Benya and Shaffer, 1982). While established cell lines and cells of animal origin tend to behave highly reproducibly in vitro, articular chondrocytes derived from adult humans have high donor to donor variability in their proliferation rate (Barbero et al., 2004), the amount of ECM produced (Barbero et al., 2004), and gene expression profiles (Grogan et al., 2007). Taken together, it may be that the inter-donor variation in types of ECM proteins synthesized, produced in varying amounts, also tend to confound the relation between cell counts and heat flow data. Although age-related changes have been reported to contribute to HAC variability (Barbero et al., 2004), no trends could be established between the donor age and the relation between μ_Q and μ_G.

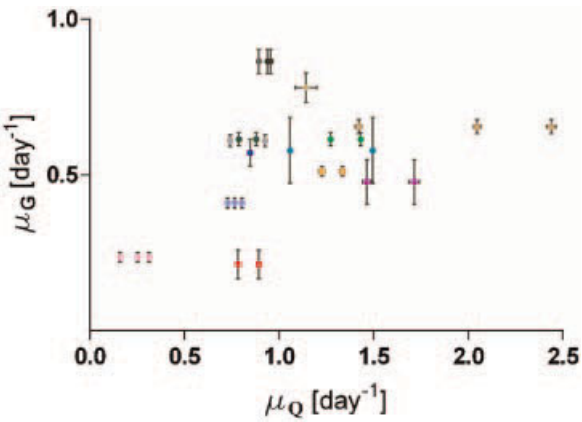

Figure 4. Growth rates (μ_G) of HAC derived from 12 different donors plotted against the corresponding heat increase rates (μ_Q). Although linear relationships have been established between μ_G and μ_Q for organisms such as bacteria with highly reproducible growth patterns, no such relationship could be established for chondrocytes derived from different human donors ($r^2 = 0.15$, $P = 0.00063$). Independent experiments are identified by different colors.

In vivo, HAC are embedded within a dense extracellular matrix and have highly restricted motility. In contrast, during in vitro culture, HAC are highly motile (mean speed of $\approx$10 μm/h) and migrate randomly or in direct response to chemotactic signals (Maniwa et al., 2001), at rates also shown to have age-related variation (Hidaka et al., 2006). While microcalorimetry has been used to assess the heat output associated with the motility of sperm (Antonelli et al., 1991) and parasitic worms (Manneck et al., 2011), no studies to date have attempted to uncouple migration-associated heat flow from other cellular energetic pathways for adherent cell types such as chondrocytes. Considering that IMC simply provides a net signal of heat flow, encompassing all chemical and physical processes, it remains a significant challenge to carefully design hypothesis driven experiments allowing to uncouple the different metabolic processes.

In this study, we established a method for the real-time monitoring of heat production by human chondrocytes during in vitro expansion. For HAC derived from particular donors, thermograms appeared to be related to the growth of the cells. However, likely due to high donor-to-donor variability among other metabolic processes occurring in parallel with proliferation, it was not yet possible to establish a predictive correlation between the heat increase rate and the growth rate, such as those readily shown for organisms with highly reproducible growth patterns (e.g., bacteria). Reproducible chondrocyte cell lines, which mitigate inter-donor variability, could be used within a more limited but more controlled model system to better understand the metabolic heat production associated with specific cell functions before adding the complexity associated with primary human chondrocytes.

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