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ORIGINAL CONTRIBUTION

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Effects of adipocyte-secreted factors on cell cycle progression in HT29 cells

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Abstract *Background* Obesity is a chronic sub-inflammatory condition which is a risk factor for several cancer diseases, e.g. colon cancer. Adipose tissue secretes biologically active factors like leptin with a known pro-inflammatory or mitogenic activity. Both, chronic inflammation and an increased cell proliferation are considered to play an important role in colon carcinogenesis. Diverse phytochemicals were shown to have cell growth inhibiting effects. *Aim of the study* The aim was to investigate whether adipocytes could mediate a proliferative capacity to HT29, a human colon adenocarcinoma cell line, and whether phytochemicals could modulate this effect. *Methods* Infranatants of adipocyte cultures from different donors were prepared and the effects of those conditioned adipocyte media (CAM) on HT29 cell growth were measured. Additionally, cell cycle progression was analyzed by flow cytometry after CAM treatment and ERK 1/2 phosphorylation was analyzed. *Results* CAM from a subgroup of adipose tissue donors stimulated HT29 cell growth, whereas others did not. This effect seems to be mediated via the ERK 1/2 pathway. Furthermore, CAM caused changes in cell cycle distribution with a shift of HT29 cells from G1- into the S-phase. This effect could be mimicked by leptin (1 nM). Co-incubation of CAM-treated HT29 cultures with β-carotene or EGCG did not have a significant impact on cell cycle progression, whereas genistein (30 μM) tended to inhibit the CAM-stimulated transition of cells into the S-phase. *Conclusion* This study confirmed the mitogenic activity of leptin in HT29 cells, although leptin secretion from adipocytes is not likely to be responsible for CAM-stimulated cell growth in our test system. The investigated phytochemicals seem to have only a minor influence on CAM-mediated cell cycle progression.

Key words colon cancer – adipokines – adipose tissue – secondary plant metabolites – proliferation

Abbreviations ANOVA: Analysis of variance, AMPK: Adenosine monophosphate-activated protein kinase, BMI: Body mass index, PKB/Akt: Protein kinase B, BHT: Butylhydroxytoluene, BSA: Bovine serum albumin, CAM: Conditioned adipocyte media, d: Diameter, DMEM: Dulbecco's modified Eagle's medium, DMSO: Dimethylsulfoxide, EGCG: (-)-Epigallocatechingallate, EGF: Epidermal growth factor, ERK: Extracellular regulated kinases, FCS:

Fetal calf serum, FITC: Fluorescein isothiocyanate, IGF: Insulin-like growth factor, IL: Interleukine, JNK: Janus kinase, MAPK: Mitogen-activated phosphokinase, KRP: Krebs-Ringer phosphate buffer, OD: Optical density, PI: Propidium iodide, PI3K: Phosphatidyl-inositol 3-kinase, PBS: Phosphate buffered saline, THF: Tetrahydrofurane, w/o: Without, WST-1: (4-[3-(4-iodophenyl-)2-(4-nitrophenyl)-2H-5-tetrazolio-]1, 3-benzodisulfonate

Introduction

Obesity is associated with an increased risk of various diseases, including certain malignancies like colon cancer (relative risks 1.2–2.0, reviewed in [19]), although the causal relationship is not yet fully understood. Elevated insulin concentrations commonly observed in obesity (reviewed in [9]) may stimulate proliferation and reduce apoptosis in colon cancer cell lines and in animal models. Additionally, alterations in the insulin-like growth factor (IGF) system adversely effects cellular growth pathways in insulin resistant state [9].

Moreover, during recent years, adipose tissue was found to represent an active endocrine organ which secretes different bioactive products collectively termed adipokines. A variety of studies have suggested that the link between obesity and colon cancer might be explained by these adipokines [10] of which adiponectin and leptin are the most abundantly secreted products. Beside control in energy metabolism and food intake leptin has been shown to stimulate growth of normal and neoplastic colonic cells [10, 12]. In turn, adiponectin is considered to demonstrate an anti-inflammatory adipokine and it's expression was shown to be reduced in the obese state [3]. Interestingly, adiponectin seems to exert pro-proliferative action on HT29 human colon cancer cells [20]. Other adipokines, cytokines and chemokines, with a known immunmodulatory function [11, 30] may additionally increase the risk for colon cancer. Especially IL-6 seems to play a crucial role for the growth of primary and metastatic carcinoma cells [5, 26].

In contrast to a high energy intake, consumption of fruits and vegetables may protect against certain types of cancer, including colorectal carcinoma [23, 34], although controversial data exist [8, 18]. Food contents like carotenoids like β-carotene [6, 21] and the polyphenol (-)-epigallocatechingallate (EGCG) may exert anti-proliferative or pro-apoptotic effects on colonic cells [14, 27] as underscored by animal models of colon carcinogenesis [35].

Aim of this study was (I) to investigate whether secretory factors from adipocytes can confer an elevated capacity for cell proliferation on intestinal epithelial cells and (II) to address potential molecular mechanisms. Additionally, we (III) were interested to investigate whether phytochemicals are able to modulate a potential adipocyte-mediated effect on the HT29 proliferation. Therefore, conditioned media from cultured human adipocytes were prepared and their effects on HT29 cells were investigated in vitro either alone or in combination with phytochemicals.

Materials and methods

Chemicals

β-carotene was pre-dissolved in THF stabilized with 0.025% BHT. Stock solutions of EGCG and genistein were prepared in DMSO. Final concentrations of the solvents in cell culture media were always 0.5%. All other chemicals were from Sigma-Aldrich (Taufkirchen, Germany) or Merck (Darmstadt, Germany). Genistein was from Extrasynthese (Genay, France), collagenase from Biochrom (Berlin, Germany).

Donors of adipose tissue samples

For the different experiments in total subcutaneous adipose tissue samples (50–100 g wet weight) were obtained from 36 (9 male, 27 female) subjects undergoing elective plastic abdominal surgery. The mean age of the donors was 45 ± 13 (range 27–82) years and their average BMI was 27.1 ± 5.3 kg/m^2. They were all of Caucasian origin and did not suffer from acute infections. The protocol was approved by the ethical committee of the Technische Universität München.

Isolation and culture of fat cells

Adipose tissue samples were transported to the laboratory in DMEM/Ham's F12 (1:1 w/w) containing 50 µg/ml gentamycin and were dissected within 1 h after operation [29]. For preparation of mature fat cells minced tissue was digested with 100 U/ml collagenase and 4% BSA for 60 min at 37°C in a shaking

water bath. Floating mature adipocytes were purified by 2 filtration steps with 2,000 and 250 μm pore size (VWR, Darmstadt, Germany) and mean cell volumes were calculated from the diameter using the formula $4/3 \times r^3 \times \pi$. The floating adipocytes were washed three times with KRP containing 0.1% BSA. For the preparation of conditioned adipocyte media (CAM) packed fat cells were resuspended with DMEM/F12 medium (1:8) supplemented with 50 μg/ml gentamycin for 16 h in a humidified atmosphere with 5% CO_2 at 37°C. Thereafter, infranatants were harvested and stored at 4°C not longer than 3 h until used in subsequent experiments. For cell viability lactate dehydrogenase (LDH) activity was measured [33]. Only cell preparations below a certain threshold were used for the experiments.

Culture of HT29 cells and proliferation assays

For the determination of proliferation HT29 human colon adenocarcinoma cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, passages 13–26) were used. The cells were cultivated in DME-medium (25 mM glucose) containing 10% (v/v) FCS, 50 units/ml penicillin and 50 μg/ml streptomycin (referred as HT29 medium). Cells were seeded into 96-well plates (5,000 cells/well) and were incubated with HT29 cell culture to allow cell attachment. After 12 h cells were incubated with test media consisting of HT29 medium and fat cell medium (→ CAM control) (1:1 (v/v)), adipocyte infranatants (→ CAM) or leptin (1 nm) containing a final concentration of 5% FCS in every medium. After 5 days in the respective medium, viability of the cells was determined using a WST-1 test (final concentration of WST 10%).

Measurement of adipokines by ELISA

Adiponectin and leptin in the cell culture media were measured by ELISAs from R&D Systems (Wiesbaden, Germany). The detection limits for adiponectin and leptin were 3.9 ng/ml and 15.6 pg/ml, respectively. The intra-assay coefficient was <5%, the inter-assay coefficient <10%. The amount of protein in the medium was normalized for 10^6 cells.

Measurement of ERK 1/2 (p42/p44) MAPK activation

Mitogen activated phosphokinases (MAPKs) ERK 1/2 (p44p42) were measured with a bead-based multiplex assay (Bio-Plex 200 System, Bio-Rad, Hercules, CA, USA). Therefore, cells CAM were added to HT 29 (5×10^3 cells/cm^2) at a dilution of 1:2 in DMEM without FBS for different time periods as indicated in the figure. Subsequently, cells were harvested in cell lysis buffer (Bio-Rad) and total protein content was determined (BCA-assay, Promega, Heidelberg, Germany). For MAP-kinase phosphorylation 10 μg of total protein were incubated with specific phosphoantibody-coupled polystyrene beads and were detected with a biotinylated secondary antibody according to the manufacturers protocol (Bio-Rad). Fluorescence signals were corrected for total ERK1/2 for the same starting amount of protein.

Cell cycle experiments

For cell cycle experiments HT29 cells were seeded into 6-well plates (125,000 cells/well) and grown for 24 h in HT29 medium. Cell cycle was synchronized by deprivation of serum for another 24 h. Subsequently, cells were treated for 15 h with the test media described above, either with or without the phytochemicals β-carotene, EGCG or genistein. For flow cytometry analysis, cells were trypsinized. Cell viability was determined by trypan blue staining and 250,000 cells of each sample were washed twice with PBS and fixed in 70% ethanol for 1 h on ice. After washing twice with PBS, 25 μl RNAase (100 μg/ml) was added to each sample. After 5 min at room temperature, cells were stained with propidium iodide (50 μg/ml) for 25 min (15 min at room temperature and 10 min at 37°C) and placed on ice until analysis. Cell cycle distribution was measured in the LSRII flow cytometer (BD Biosciences, Heidelberg, Germany) with an argon ion laser emitting light at 488 nm. At least 10,000 cells per sample were measured and data analysis was performed with the FACSDiva™ software version 3.0. For the statistical analysis of cell cycle distribution ModFit *LT*™ Version 2.0 (Verity Software House Inc., Topsham, USA) was used.

Statistical analysis

Statistical analyses were carried out using the SigmaStat® 3.0 (SPSS Science Software, Erkrath, Germany). Differences in cell growth and cell cycle distribution between treated and corresponding control cells were tested using paired Student's *t* tests. Linear correlations between different parameters were done to see their relationships. The significance level was set at $P \leq 0.05$. If the measured data were not normally distributed, adequate nonparametric statistical tests were used. As adipose material was limited not all analyses from every sample could be performed. Therefore, number of samples is always given.

Results

Influence of conditioned adipocyte-media on HT29 cell proliferation

Thirty CAM were used for the assessment of proliferation-promoting activity in HT29 cells. Interestingly, only CAM from 11 donors led to a significant mean increase in cell growth by 22 ± 9% (Fig. 1). However, the others failed to stimulate proliferation. There was no significant effect when all samples were combined (Fig. 1). Baseline characteristics of donors from active and inactive CAM were not different (data not shown). There was no correlation of HT29 cell growth with BMI, age, and mean fat cell size of the donors. Two-way ANOVA did not show any influence of gender ($P = 0.54$) on HT29 cell growth after CAM treatment (data not shown).

Measurement of adipokines in CAM and their effects on HT29 cells growth

As shown recently, leptin caused potent proliferation in colonocytes [24]. In own experiments, use of 1 nm recombinant leptin for 5 days resulted in a significant proliferation of HT29 cells by 75 ± 39% ($P < 0.05$, data not shown). In CAM Leptin levels greatly varied from 24 to 2,200 pg/ml. Although logarithmic ($\log_{10}$) leptin concentration from all measured CAM was not significantly associated with the BMI of the adipose tissue donors ($R = 0.20$; $P = 0.49$; $n = 14$; Fig. 2a) leptin levels in the CAM samples with proliferation-stimulating effects were closely associated with donor BMI ($R = 0.82$; $P = 0.01$; $n = 8$; Fig. 2a). Hereby leptin levels in stimulating CAM were 24.7 ± 18.4 and 51.6 ± 54.5 nm, in non-stimulating CAM respectively. However, cell growth tended to be negatively correlated with leptin concentrations in CAM if all samples were analyzed ($R = -0.45$; $P = 0.08$; $n = 16$; Fig. 2b). Considering only data from CAM that significantly

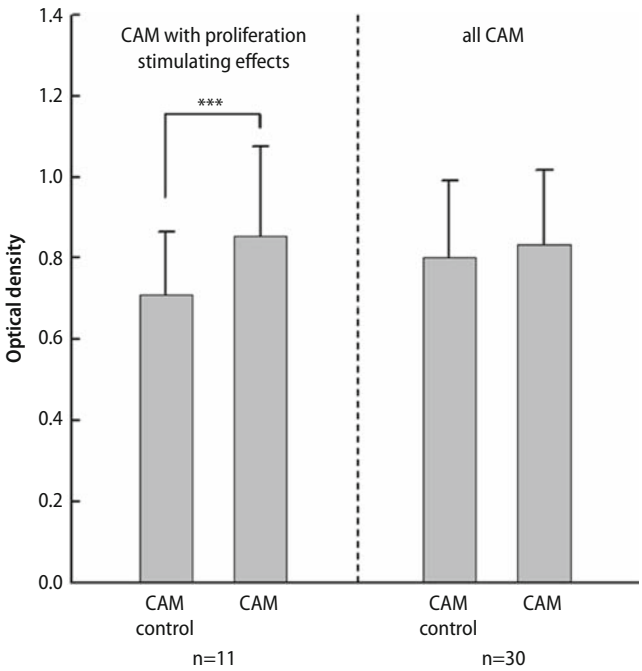

Fig. 1 Influence of conditioned adipocyte media (CAM) on cell growth of HT29 cells after a 5-day treatment measured photometrically as fomazan formation from WST-1. The *left panel* represents data from cultures significantly stimulating HT29 growth; the *right panel* includes data from all tested CAM. Data are expressed as mean values of OD (560–690 nm, blank subtracted) + SD from measurements in triplicate. The CAM controls represent cells cultivated with DMEM and DMEM/F12 (1:1 (v/v)) containing 5% FCS. ***$P \leq 0.001$ active CAM vs. corresponding CAM control, *n* number of CAM donors

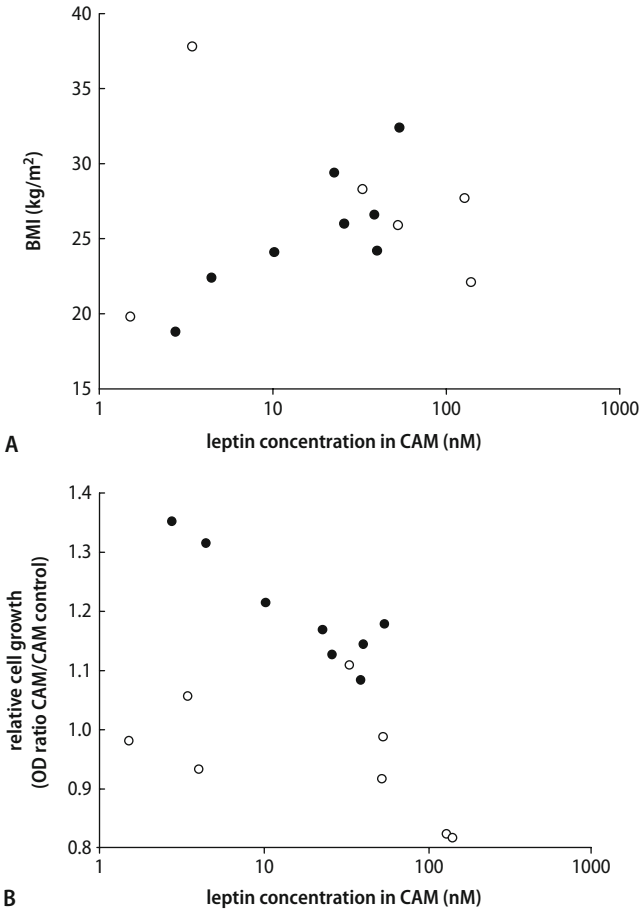

Fig. 2 Relationship between leptin concentrations in CAM and BMI of the adipocyte donors (**a**) and HT29 cell growth (**b**). Data for the leptin concentrations were logarithmically transformed to obtain a linear relationship between the two parameters. For cell growth experiments (**b**) HT29 were grown for 5 days in the presence of CAM as described in Methods. Proliferation data are expressed as ratio of mean OD values after CAM treatment related to the corresponding adipocyte media control (CAM control). *Filled circle*: Data from donors with significant proliferation stimulating effects ($n = 8$)

stimulated cell growth (black dots), the reverse correlation between leptin concentration in CAM and HT29 proliferation activity was notably high and reached statistical significance ($R = -0.91$; $P = 0.002$; $n = 8$; Fig. 2b). Measurement of adiponectin exhibited concentrations between 8.6 and 88.7 ng/ml in CAM and did not result in a significant association to cell growth ($R = -0.22$; $P = 0.43$; data not shown). Adiponectin concentrations in stimulating CAM 30.2 ± 24.3 versus 28.8 ± 17.6 ng/ml in non-stimulating CAM.

Effect of conditioned adipocytes media on MAP-kinase and I-κB phosphorylation

The ERK 1/2 (p44/p42) pathway is a major mediator for cell growth (reviewed in [1]). Therefore, CAM were added in a time-dependent manner to measure the phosphorylation of ERK 1/2, JNK, p38, and I-κB. CAM were found to stimulate phosphorylation of ERK 1/2 MAP-kinase with a maximum effect after 5 min by 5.4 ± 1.4 fold compared to control medium (Fig. 3). JNK, p38, and I-κB were not significantly affected (data not shown). To further characterize the role of ERK 1/2 MAP-kinase as a possible mediator of the effect of CAM, we exposed HT29 cells to 10 μM UO126, a specific inhibitor of MEK 1/2, 30 min. prior to the incubation with CAM. As demonstrated in Fig. 3 UO 126 did not affect basal ERK 1/2 phosphorylation, but completely inhibited the stimulatory action of CAM. Again, JNK, p38 and I-κB were not affected (data not shown).

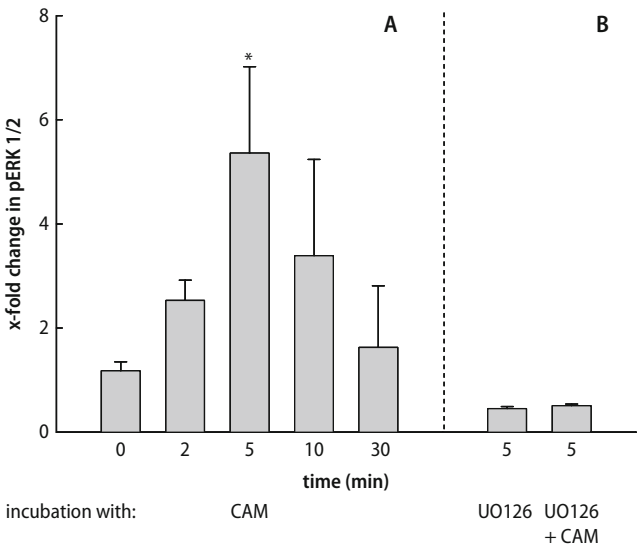

Fig. 3 Time dependent effect of CAM treatment on ERK 1/2 (p 44/42) phosphorylation in HT29 cells (**a**). Phospho-ERK 1/2 was corrected for total ERK 1/2 and data are expressed as mean fluorescence intensities in relation to its respective control. CAM were added for the time points indicated. UO126 was added at a concentration of 10 μM 60 min prior to the co-incubation with CAM for 5 min (**b**). Columns represent + SD from three independent experiments in duplicate. *$P \leq 0.05$ vs. 0 min

Effect of phytochemicals on leptin- and CAM-stimulated cell cycle progression of HT29 cells

To assess the effect of phytochemicals on leptin or CAM-stimulated HT29 cell growth, cells were deprived from FCS for 24 h to ensure a synchronization of cells. Flow cytometric cell cycle analysis showed that 87% of serum-free control cells were in the G_0/G_1-phase, 9% in the S-phase and 4% in the G_2/M-phase. A 15-h treatment of HT29 cells with leptin at a concentration of 1 nM resulted in a significant 3.5-fold increase of cells in the S-phase of the cell cycle compared to serum-free control cells. To study the effect of selected phytochemicals HT29 cells were pre-incubated with different concentrations of β-carotene (5 and 20 μM), (-)-epigallocatechingallate (EGCG) (5 and 25 μM), or genistein (10 and 30 μM) for 2 h. Then, leptin or CAM were used to induce HT29 proliferation either in the presence or absence of the respective phytochemicals. Concerning the effect of 1 nM leptin, neither β-carotene, nor EGCG nor genistein could significantly modulate the effect on cell cycle distribution (data not shown). In contrast, addition of genistein (30 μM) to CAM treated cells tended to result in an inhibition of the shift of the HT29 cells into the S-phase (Fig. 4). The percentage of genistein-treated cells in the S-phase was reduced by 13 ± 2% ($P < 0.13$). Furthermore, CAM-stimulated transition of HT29 cells into the S-phase was not affected by co-incubation with β-carotene and EGCG (Fig. 4).

Discussion

Chronic inflammation and an increased colonic cell proliferation are suggested to represent important events in colon carcinogenesis (reviewed in [4]). Obesity is a major cause for a chronic low-grade inflammatory condition and this may be one link for the association between obesity and the increased risk of colon cancer [9]. Adipose tissue could mediate proliferative signals to the colonic epithelium by the secretion of pro-inflammatory adipokines. In the present study, conditioned adipocyte media (CAM) from different adipose tissue donors were prepared and showed variable effects on HT29 cell growth. The co-incubation model in our study uses a similar approach as recently published for other cell types [28, 37]. The approach may mirror the in vivo situation at least in part as we were able to identify a variety of

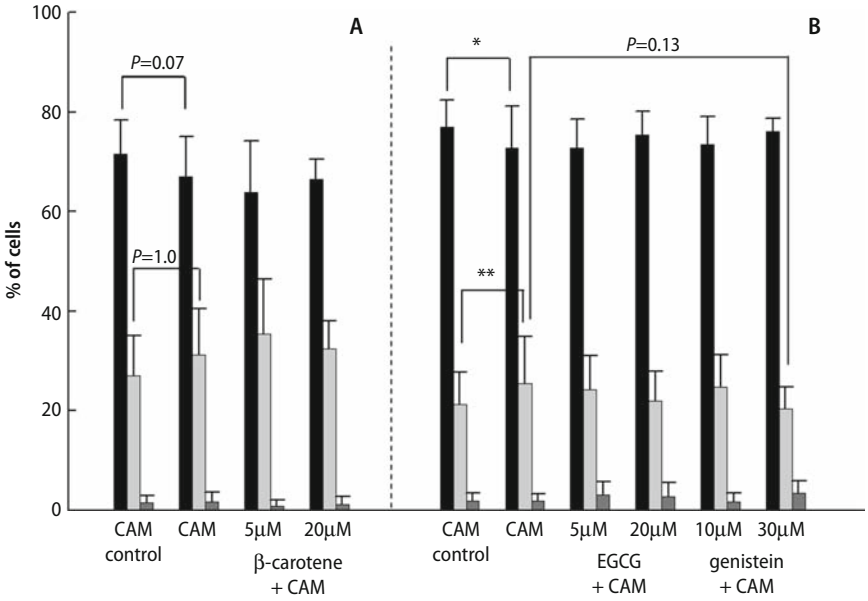

Fig. 4 Cell cycle distribution of HT29 cells after 15 h treatment with CAM and its modification by phytochemicals: *black shaded bars* G_0/G_1-phase, *light shaded bars* S-phase, *dark shaded bars* G_2/M-phase. (**a**) All media contained 0.5% (v/v) THF. (**b**) All media contained 0.5% (v/v) DMSO. Data are expressed as cell fractions (% of the total cell population) + SD of at least 5 independent experiments. $^*P \leq 0.05$, $^{**}P \leq 0.01$ vs. CAM control

adipokines which also play a role in diseases with a chronic inflammatory background like diabetes or atherosclerosis [11, 30, 31]. Although we were not able to demonstrate significant associations between BMI, fat cell volume or leptin and HT29 growth CAM from a subset of donors were identified to significantly stimulate the proliferation of the cultured cells. As conditioned medium from adipocytes demonstrates a mixture of more than 100 secreted factors the proliferative effectors which could act as a potential biomarker still have to be defined.

An interesting adipokine in this context known to stimulate cell proliferation in colon cells is leptin [16]. In line with other studies [16, 24], we observed a significant stimulation of HT29 cell growth after treatment with leptin, already at a concentration of 1 nM (16 pg/ml, data not shown) which is lower than serum concentrations in normal-weight subjects (2–13 ng/ml, [7, 13]). In the present study, leptin concentrations in CAM ranged from 24 to 2,200 pg/ml (1.5–137.5 nM) and HT29 cells were incubated with half of those concentrations due to the 1:2 dilution of CAM in HT29 medium. Nevertheless, instead of a positive association between leptin concentrations in the growth-promoting CAM and the relative increase in cell growth we found a reverse association, which may indicate that other secreted factors from adipocytes contribute to the total biological activity of the respective CAMs either alone or in combination. In animal experiments, a significant positive correlation between serum leptin concentration and colonic cell proliferation was previously shown [16]. Although our results are in line with published data showing, that adipocytes promote colonocyte cell growth [2], they also seem to contradict the significance of leptin in this respect [16, 17, 20].

We also measured adiponectin, another adipokine which was recently shown to stimulate proliferation of HT29 cells at concentrations of 0.1–5.0 µg/ml [20]. Adiponectin concentrations in CAM varied between 8.6 and 88.7 ng/ml but did not correlate significantly with HT29 cell growth (data not shown).

CAM were prepared from adipocytes of the subcutaneous depot as major adipose tissue which is representing at least 80% of total fat mass [20]. As visceral and subcutaneous adipose tissue show different secretion patterns including leptin secretion [32], additional investigations about the effects of CAM from isolated adipocytes of visceral origin on HT29 cell growth should be performed. With respect to the heterogeneous effects of CAM seen in subcutaneous adipocytes, however, more samples have to be investigated to draw final conclusions.

To get first insight into possible intracellular mechanisms responsible for the stimulation of HT29 cell growth, we measured the phosphorylation of selected signalling proteins. The mitogen-activated protein kinase (MAPK) cascade is a highly conserved pathway that plays an important role for cell proliferation, differentiation and migration. Other members of the MAPK family are ERK 1/2, also known as p44/p42 and the c-Jun N-terminal kinase (JNK). In the present study, exposure of HT29 cells to CAM resulted in a marked increase of ERK 1/2 phosphorylation. Therefore, inhibition of this pathway potentially prevents cell proliferation. There are great efforts to identify naturally occurring products to inhibit stress-activated protein/MAPK pathways such

as resveratrol, a product of red wine [15]. Phytochemicals like carotenoids and polyphenols are suggested to considerably contribute to the protective effects of fruits and vegetables against colorectal cancer due to their marked anti-proliferative effects on HT29 cells accompanied by induction of cell cycle arrest and apoptosis [6, 14]. There is also some evidence that the green tea polyphenol EGCG inhibits ERK 1/2 activation [25].

In our cell cycle experiments, we investigated the effect of the phytochemicals on HT29 cell cycle progression as previously described in the literature [22, 36]. An incubation time of 16 h was chosen to minimize cell fragmentation due to treatment-induced apoptosis. The phytochemicals β-carotene and EGCG were not found to modulate the effect of leptin and CAM on cell cycle distribution of serum-deprived cells significantly (Fig. 4). At least, genistein at a concentration of 30 μM tended to inhibit the shift of cells into the S-phase when they were co-incubated with CAM for 15 h (Fig. 4).

In conclusion, the present study provides first evidence that CAM prepared from adipocytes of subcutaneous adipose tissue from human donors exerts some potential to stimulate cell growth of HT29 cells, although this effect was not observed in the media from all donors. As leptin concentrations in CAM were negatively correlated with HT29 cell growth other adipokines may mediate this effect. Therefore, future studies should focus on factors with a pro-proliferative potential which could serve as biomarkers. Phytochemicals used in the present in vitro study had only a minor if not negligible influence on CAM-mediated proliferation only genistein exhibited a modest anti-proliferative effect. Nevertheless, our cell culture model using primary fat cells, which retain to a certain degree the phenotype of the individual donor may be suitable to gain insight into the complex interplay of mediators which contribute to the development of colon cancer in obesity.

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