

Influence of zinc and zinc chelator on HT-29 colorectal cell line

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Abstract Trace elements are involved in many key pathways involving cell cycle control. The influence of zinc and zinc chelator (TPEN) on transcription levels of the main zinc transporters (ZnT1 and ZIP1) in the HT-29 colorectal cell line has not been reported. Proliferation of HT-29 cells was measured using the methylene blue assay after exposure to zinc (two concentrations), TPEN (two concentrations), or a combination of zinc and TPEN (simultaneously and sequentially) for 4 h, 8 h, and 24 h. The transcription levels of ZnT1, ZIP1, vascular endothelial growth factor (VEGF), and caspase-3 were determined using

reverse transcriptase real-time polymerase chain reaction (RT-PCR) after exposure of cells to zinc and TPEN. The zinc content in the substrate (medium used for culture) was determined using atomic absorption spectrometry. TPEN decreased cellular proliferation causing complete cell death by 8 h. Zinc had a protective effect against short periods of exposure to TPEN. There was no correlation between the transcripts of main zinc transporters and the zinc content in the substrate. The zinc content in the substrate remained constant after varying periods of cell culture. TPEN decreased the transcript levels of caspase-3 and VEGF, which are surrogate markers for apoptosis and angiogenesis. Zinc chelation of HT-29 cells causes cell death. Zinc appears to be protective for short periods of exposure to TPEN but has no protective effect on prolonged exposure. HT-29 cells are not able to counteract the effect of intracellular chelation of zinc by altering zinc transport. Further research into the mechanisms of these findings is necessary and may lead to novel therapeutic options.

Keywords Zinc · Zinc chelator · Angiogenesis · Apoptosis · HT-29

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Background

Colorectal cancer is the third most common malignancy in the UK with an annual incidence of 35,500

patients (Westlake 2008). Colorectal cancer is the second leading cause of cancer mortality, next only to lung cancer. It accounts for nearly 10% of all cancer deaths (Westlake 2008) and for 1 in 30 deaths due to all causes (National Statistics 2008; Westlake 2008) resulting in nearly 16,000 deaths in the UK (Westlake 2008). The liver is the most common site of recurrence in people who undergo curative surgery for colorectal cancer (Goldberg et al. 1998; Tepper et al. 2003; Cho et al. 2007). Surgical resection is generally considered the best option in patients in whom surgical resection is possible (Khatri et al. 2007).

Trace elements are involved in many key pathways involving cell cycle control. Mobilisable iron increases the production of oxygen free radicals (Barbouti et al. 2001) and promotes tumorigenesis (Rezazadeh and Athar 1997; Rezazadeh et al. 2005). Mobilisable copper also causes oxidative damage (Ferretti et al. 2003) and promotes angiogenesis (Finney et al. 2009; Xie and Kang 2009). High levels of zinc induce apoptosis (programmed cell death) in prostate cells (Costello and Franklin 2006). Zinc is a co-factor or an essential component of nearly 300 enzymes including metallothionein, superoxide dismutases and plays a role in anti-oxidation, immune function and inflammation (Tapiero and Tew 2003). Adequate levels of zinc are required for the nuclear functions during early differentiation (Beyersmann and Haase 2001).

Iron, copper, and zinc are lower in colorectal liver metastases than in normal surrounding liver (Gurusamy et al. 2008; Farquharson et al. 2009). The transcription levels of the main zinc efflux transporter ZnT1 is consistently low in colorectal liver metastases compared to normal surrounding liver (submitted for publication). The main zinc influx importer (ZIP1) was low in colorectal liver metastases compared to normal surrounding liver. However, the magnitude (there was only a 1.25-fold decrease) and the consistency of this decrease are poor (submitted for publication). The reason for this combination of low-zinc content in combination with a low-zinc efflux transporter could be increased zinc utilisation by cancer cells. This study was performed to investigate this process further by determining the influence of zinc and zinc chelator, *N,N,N',N'*-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), on cell proliferation and the transcription levels of the main

zinc influx and efflux transporters, i.e. ZIP1 and ZnT1. TPEN is generally considered a membrane permeable selective intracellular zinc chelator (Kolenko et al. 2001; Kindermann et al. 2004). This might give a clue as to whether the main zinc influx and efflux transporters were altered as a result of the altered levels of zinc or whether they were the cause of the altered levels of zinc.

Caspase-3 activation is one of the key mechanisms of programmed cell death or apoptosis (Porter and Janicke 1999). Decrease in cell apoptosis may promote tumour growth. Vascular endothelial growth factor (VEGF) plays an important role in the induction of tumour angiogenesis (new vessel formation) and hence tumour growth (Carmeliet 2005). Measuring the transcription levels of caspase-3 and VEGF may provide clues to the mechanism of action of zinc chelators.

Methods

Cell culture

HT-29 colorectal cell line (ECACC, Salisbury, Wiltshire, UK) between passages 140 and 170 was used for this research. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l glucose with L-glutamine (Lonza, UK) with 10% foetal calf serum (Sigma-Aldrich, UK) and gentamycin 50 µg/ml (Sigma-Aldrich, UK). Cells were sub-cultured at 60–70% confluence using trypsin/EDTA at 1 mg/ml concentration (both trypsin and EDTA from Lonza).

Experimental groups

Zinc in the form of a solution of 0.1 M zinc chloride (Sigma-Aldrich, UK) was diluted in serum free DMEM medium (Lonza, UK) to obtain concentrations of 8 µmol/l ('low-zinc-supplement') and 16 µmol/l ('high-zinc-supplement'). Zinc chelator, TPEN (Sigma-Aldrich), was used diluted in DMEM medium to obtain concentrations of 4 µmol/l ('TPEN-low') and 8 µmol/l ('TPEN-high'). The choice of the concentration was based on a previous report (Kindermann et al. 2004) and preliminary dose response curve experiments which

were performed to detect a discernible difference in the proliferation.

Cell proliferation

For cell proliferation assays, cells were grown in 96 well microplates. A volume of 100 μ l containing 20,000 cells were grown in the above medium for 24 h before exposure to the 100 μ l of the following groups: low-zinc-supplement, high-zinc-supplement, TPEN-low, TPEN-high, a combination of zinc and TPEN at each of high and low doses resulting in four combinations (low-zinc-supplement + TPEN-low; low-zinc-supplement + TPEN-high; high-zinc-supplement + TPEN-low; and high-zinc-supplement + TPEN-high), sequential exposure of the cells to zinc and TPEN at different doses and sequences resulting in eight combinations (four groups with exposure to zinc first for the first half of the total period of the assay and TPEN for the second half of the total period of the assay; four more groups with exposure to TPEN first followed by exposure to zinc), and the control group of DMEM. The seeding concentration and time of assay was chosen based on preliminary experiments. Cell proliferation was determined using methylene blue assay (Oliver et al. 1989) using Biotek plate reader at 630 nm wavelength. The results of cell proliferation are expressed in arbitrary calorimetric units (AU). The experiment was repeated after altering the well positions of the different groups in the plate.

RNA extraction

For assessment of transcription levels, 25 ml culture flasks were used for cell culture. The cells were grown in a volume of 5 ml of the above medium till 60–70% confluence was reached before exposure to 5 ml of different groups (low-zinc-supplement, high-zinc-supplement, TPEN-low, TPEN-high, and DMEM). The time of assay was chosen based on preliminary experiments. At the chosen time of the assay, the cells were trypsinized using the same trypsin solution used for splitting, collected by centrifugation at 1,500 $\times$ g, washed with phosphate buffered saline (PBS), and stored at -80°C until extraction of RNA. RNA was extracted using RNeasy Mini Kit (Qiagen, UK). The extracted RNA was stored at -80°C until conversion to complementary DNA (cDNA).

Measurement of transcription levels

One microgram (1 μ g) of total RNA was reverse-transcribed to cDNA using the QuantiTect Rev. Transcription Kit (Qiagen, UK). The relative transcription levels were measured by real-time polymerase chain reaction (RT-PCR) using Lightcycler II (Roche, USA). The composition used for the Lightcycler reaction was 10 μ l of Quantifast SyBrGreen (Qiagen, UK), 2.5 μ l of forward primer (0.5 μ mol), 2.5 μ l of reverse primer (0.5 μ mol), 4 μ l RNase free water (Qiagen, UK), and 1 μ l cDNA. The forward and reverse primers were each of 20 base pairs and were designed using the free primer design software Primer3-Web 0.4. An amplicon size of 150 and 200 base pair (bp) was used (Fleige et al. 2006). The standard Lightcycler protocol was used for amplification of cDNA (for quantification of RNA) and for melting curve analysis (for identification of the specificity of the primers used) without any modification. ‘Fit-point method’ was used for RNA quantification (LightCycler 2000; Pfaffl 2001). The threshold cycle to cross a pre-defined fluorescence level was obtained. Relative quantification of mRNA levels was determined by adjusting for beta-actin-1 using the method described by Pfaffl (2001). The specificity of the PCR product was assessed by melting curve analysis. To carry out melting curve analysis, the temperature was increased very slowly from a low temperature (65°C) to a high temperature (95°C) using the Lightcycler. At low temperatures, all PCR products are double stranded, so SYBR Green I bind to them and fluorescence is high, whereas at high temperatures, PCR products are denatured, resulting in rapid decreases in fluorescence (LightCycler 2000). The fluorescence was measured continuously as the temperature was increased and plotted against temperature. A curve is produced by plotting the fluorescence against temperature, because fluorescence decreases slightly through the lower end of the temperature range, but decreases much more rapidly at higher temperatures as the melting temperatures of nonspecific and specific PCR products are reached (LightCycler 2000). The melting curve analysis for the transcription levels demonstrated a single peak indicating that the PCR product was specific. This showed that there were little or no impurities in the PCR product indicating that the fluorescence measured reflects the

transcription levels of the gene and not non-specific products or impurities.

Substrate utilisation

The medium from the cell cultures used for measuring the transcription levels (before the cells were trypsinized) was centrifuged to remove any cells. The supernatant devoid of any cells was used for measuring the zinc content using atomic absorption spectrometry in order to determine the zinc utilisation by the cells.

Validity of the results and statistical analysis

All the experiments were repeated to confirm the consistency of the results. Statistical tests were not performed as the experiment was performed only twice with multiple replicates within each run. The correlation between ZnT1 and ZIP1 was determined using spearman correlation coefficient using StatsDirect statistical software version 2.7.

Results

Cell proliferation

The proliferation of cells cultured in the presence or absence of zinc or TPEN or a combination of the two is shown in Fig. 1. At 4 h, a trend towards lower proliferation in the groups exposed to TPEN either alone or in a sequential combination with zinc was noted. The lower proliferation appeared to be dependent on the concentration of TPEN for the TPEN alone groups but not for the sequential combination groups. The trend of lower proliferation was not noted in cells exposed to a simultaneous combination of zinc and TPEN. At 8 h, the cells exposed to zinc had similar proliferation as normal cells. The cells exposed to TPEN had died by 8 h (both in the low and high concentrations). Sequential exposure to zinc and TPEN showed that exposure to TPEN at higher concentrations resulted in lower proliferation than TPEN at lower concentration or controls, although the effect seems to be greater in the groups where the TPEN was used second. A combination of zinc and

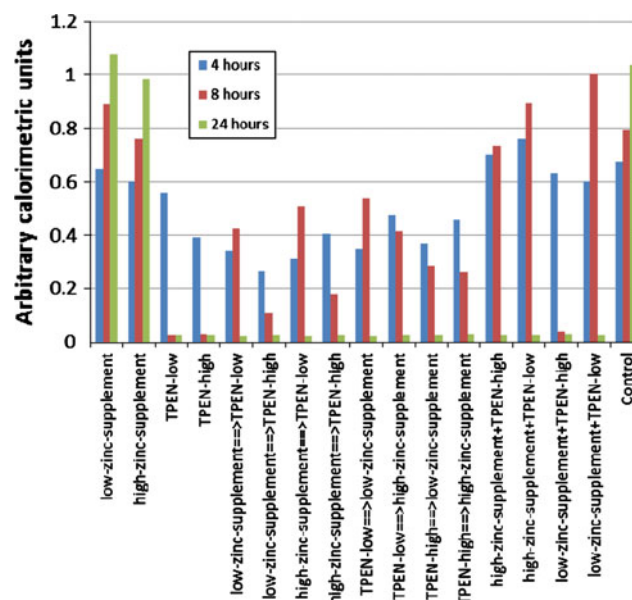


Fig. 1 Proliferation in different groups. The cell proliferation at different time periods (4 h, 8 h, and 24 h) expressed in arbitrary calorimetric units (AU). The experiment was repeated and showed the consistency of the results. Sequential exposure to the solutions is indicated by the symbol '==>'. The cells were exposed to the first solution (to the left of the symbol '==>') for

half the total duration, i.e. 2 h for the 4 h group; 4 h for the 8 h group; and 12 h for the 24 h group. After this 'half-time', the cells were exposed to the second solution (to the right of the symbol '==>') for the remaining duration. Simultaneous combination is indicated by the symbol '+'

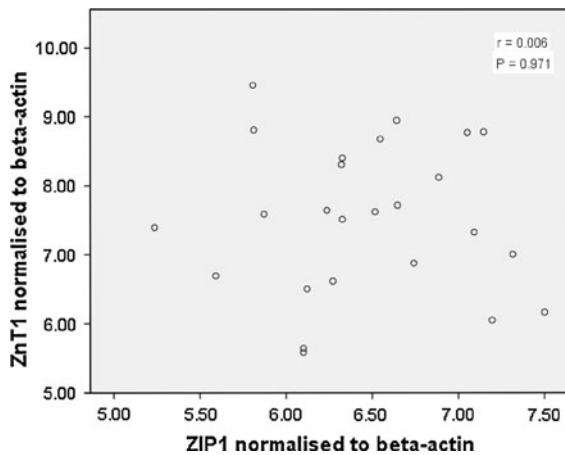


Fig. 2 Scatter plot of ZnT1 versus ZIP1 in HT-29 cell line. r = correlation coefficient. There was no statistically significant correlation between ZIP1 and ZnT1. ZnT1 and ZIP1 were normalised to beta-actin levels

TPEN for the entire period of 8 h shows that the cells were surviving at 8 h in all groups other than the combination of low-zinc with high TPEN. By 24 h, irrespective of the dose of TPEN used and whether it was used in combination with zinc or not, the cells had died. These results were reproducible on repeat experiments.

Transcription levels and substrate utilisation

The transcription levels of any of the genes could not be determined after 8 h and 24 h of exposure to TPEN because this treatment killed all cells. Measurement of the relative transcription levels under other experimental conditions could be determined. There was no consistent pattern of ZnT1 and ZIP1 transcript levels at any point of time (4 h, 8 h, or 24 h). The scatter plot of ZnT1 and ZIP1 transcript levels does not show any correlation between ZnT1 and ZIP1 (Fig. 2). The correlation co-efficient was -0.31 ; $P = 0.2535$.

The zinc content in the medium without any cell culture was $2.8 \mu\text{mol/l}$. The zinc content in the supernatant fluid from the cell cultures used for measuring the transcription levels and the relationship between the zinc content in the supernatant and the transcription levels of ZnT1 and ZIP1 are shown in Fig. 3a, b. There was no correlation between the zinc concentration and ZnT1 or ZIP1 transcript levels.

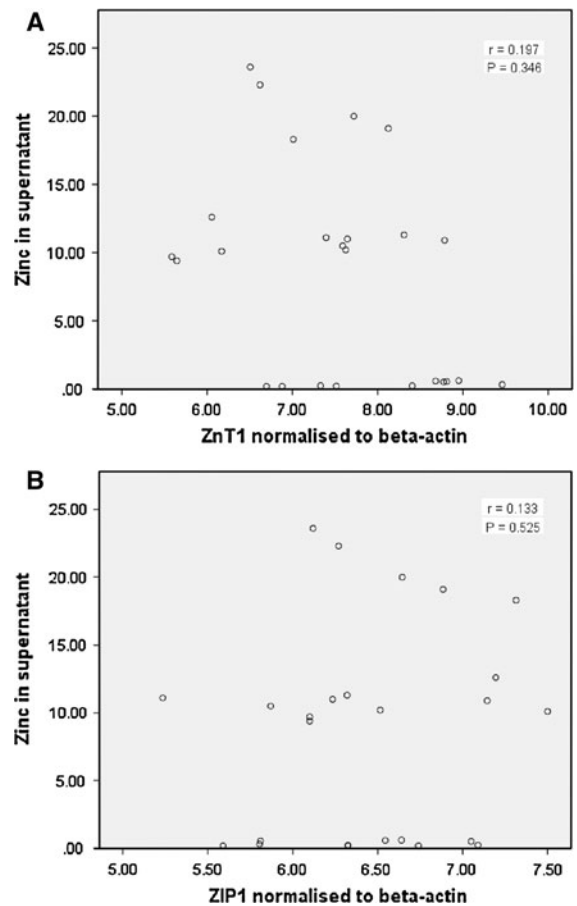


Fig. 3 **a** Scatter plot of zinc ($\mu\text{mol/l}$) versus ZnT1 in HT-29 cell line. **b** Scatter plot of zinc ($\mu\text{mol/l}$) versus ZIP1 in HT-29 cell line. r = correlation coefficient. There was no statistically significant correlation between zinc, ZIP1, and ZnT1. ZnT1 and ZIP1 were normalised to beta-actin levels

The zinc levels were seen in three clusters. The lowest cluster corresponded to controls (cell cultures grown in serum free DMEM) and TPEN groups. This level was similar to the level seen in serum free DMEM medium not used for culture. The middle cluster corresponded to the groups with lower level of zinc. This level was similar to the level seen in low-zinc-supplement medium ($8 \mu\text{mol/l}$) not used for cell culture. The highest cluster corresponded to the groups with higher level of zinc. This level was similar to the level seen in high-zinc-supplement medium ($16 \mu\text{mol/l}$) not used for cell culture. There was no significant change in the zinc level with time.

In order to determine the cause of cell death in the TPEN group at 8 h and 24 h, relative transcription levels of VEGF (vascular endothelial growth factor or

Table 1 Relative transcription levels of VEGF (angiogenesis factor) and caspase-3

Gene	TPEN (low) Mean (95% CI)	TPEN (high) Mean (95% CI)
VEGF	0.339 (0.193–0.483)	2.09 (0.222–3.967)
Caspase-3	2.514 (0–6.026)	0.398 (0.202–0.595)

* In comparison with DMEM as calculated by the method described by Pfaffl (2001)

Mean and 95% confidence intervals (CI) were calculated by repeating the experiment twice. A value of less than one indicates that the transcription level in TPEN is lower than that in control. A value of greater than one indicates that the transcription level in TPEN is greater than that in control. If the confidence intervals overlap a value of one, it indicates that the actual relative transcription levels estimated from the values estimated from the experiment could either be increased or decreased

angiogenesis promoting factor) and caspase-3 were determined. The relative transcription levels of VEGF and caspase-3 in the TPEN (low) and TPEN (high) in comparison with control (DMEM) are shown in Table 1. From the estimates obtained from this experiment, it appears that TPEN at low concentrations suppressed VEGF. TPEN at higher concentrations suppressed caspase-3. There was no difference in the caspase-3 or VEGF expressions in the zinc groups (not shown).

Discussion

This study was performed to determine the influence of zinc and zinc chelator TPEN on cell proliferation and the transcription levels of the main zinc influx and efflux transporters, i.e. ZIP1 and ZnT1. TPEN is generally considered a membrane permeable selective intracellular zinc chelator and its main actions are believed to be due to its effect on zinc (Kolenko et al. 2001; Kindermann et al. 2004).

Proliferation

Proliferation was measured by methylene blue assay, which shows the viable cells. At 4 h, a trend towards lower proliferation in the groups exposed to TPEN either alone or in a sequential combination with zinc was revealed. A lower proliferation may indicate lack of cell division (mitosis) or increased cell death. A period of 4 h is not enough to induce new protein

synthesis and this induced protein cause noticeably lower proliferation. However, a lower proliferation was noted after just 4 h of exposure to TPEN. The lower proliferation could be because TPEN chelates zinc thereby affecting the activity of essential enzymes which need zinc as co-factor. The lower proliferation could also be because of mitochondrial permeability transition (MTP) (increase in mitochondrial permeability) (Hunter et al. 1976) with a consequent alteration in the apoptosis or necrosis (Kroemer et al. 1998). However, in the current study, the caspase-3 transcript level was decreased after exposure to TPEN for 4 h suggesting that apoptosis induced by MTP is unlikely to be the cause for lower proliferation. An alternative explanation could be phosphorylation of the protein kinases involved in cell proliferation, which increase the proliferation of the cells without the necessity for synthesis of new proteins (Gao and Xing 2009). The lower proliferation does not seem to be due to direct toxicity as the values for proliferation are similar with low and high TPEN levels when used in combination with zinc.

At 8 h, the cells exposed to TPEN alone had died. Sequential exposure to TPEN and zinc (exposure to each group for 4 h) showed that a dose dependent effect of TPEN in lowering proliferation. This suggests that zinc rescues cell growth after a brief period of exposure to TPEN. However, preloading the cells with zinc appears to be less beneficial than providing zinc after exposure to TPEN.

By 24 h, irrespective of the dose of TPEN used and whether it was used in combination with zinc or not, the cells had died. This cell death at 24 h would suggest that the protective effect of zinc in the simultaneous exposure of cells to zinc and TPEN at 4 and 8 h is because of an active metabolic process. Another point to be noted is that the cells exposed to a simultaneous combination of zinc and TPEN-low for 8 h had normal proliferation whereas they were dead after exposure to TPEN for 24 h. One possible reason for these observations is that the permeability of the cell membrane to TPEN and zinc was different. However, if this was the case, then a difference in proliferation should have been noted even at 8 h for the simultaneous combination of zinc and TPEN (cells exposed to TPEN alone were all dead by 8 h). However, no such difference was noted except for the combination of low-zinc-supplement and TPEN-high. An alternative explanation to differential cell

membrane permeability to zinc and TPEN could be that TPEN triggers the activation of a mechanism involving protein synthesis (indicated by the delay in the death of cells exposed to zinc and low concentrations of TPEN) in addition to its early effect of decreasing proliferation by affecting essential pathways involving zinc as co-factor or by other possible mechanisms such as mitochondrial permeability or protein kinases involved in cell proliferation. Thus, there is currently no information on the reasons for the two main observations noted in the study, i.e. the early effect of zinc chelator reversed by zinc administration and late effect of the zinc chelator unaffected by zinc administration.

Transcription levels and substrate utilisation

ZnT1, ZIP1, and zinc

There were no differences in the relative transcription levels of ZnT1 or ZIP1 in the different groups at any time. There was also no significant change in the zinc content in the medium used for cell culture after growing cells for various periods of time, i.e. the control (serum free DMEM) and the TPEN group had zinc levels similar to serum free DMEM (which was at the lowest detection limit for zinc by the method used for determining zinc in this research); the low-zinc-supplement group (either alone or after a period of exposure to TPEN-low) had zinc levels similar to unused 8 µmol/l zinc in serum free DMEM; the high-zinc-supplement group had zinc levels similar to unused 16 µmol/l zinc in serum free DMEM at all time points. There was no correlation between ZnT1, ZIP1, or zinc in the supernatant. Another study on the role of TPEN on HT-29 cells using micro-array methods also did not find any differences in the transcription levels of the major zinc transporters (Kindermann et al. 2004). These findings suggest that the HT-29 cells are not able to counteract the effect of intracellular chelation of zinc by increasing the import of zinc into the cell or by decreasing the export of zinc from within the cell.

Apoptosis and angiogenesis pathways

In order to determine the mechanism of the decrease in cell proliferation, the transcription levels of

caspase-3 and vascular endothelial growth factor (VEGF) were determined. Caspase-3 activation is one of the key mechanisms of apoptosis (Porter and Janicke 1999). The caspase-3 transcription levels were lower in the TPEN-high group. This suggests that apoptosis induction via caspase-3 is unlikely to be the mechanism of the decreased cell proliferation by TPEN. However, since the main action of TPEN is chelating zinc (Kolenko et al. 2001; Kindermann et al. 2004), it suggests that zinc may induce apoptosis. This finding appears to support our observation of lower zinc content in colorectal liver metastases than normal liver (i.e. cancer would have lower apoptosis and is associated with lower zinc). Previous experimental studies on other models have shown conflicting relations between zinc and apoptosis (Min et al. 2007; Zhou et al. 2008).

VEGF plays an important role in the induction of tumour angiogenesis and hence tumour growth (Carmeliet 2005). The VEGF transcription levels were lower in the TPEN-low group compared with DMEM (controls). This suggests that zinc promotes angiogenesis. Previous experimental studies on other models have again shown conflicting relations between zinc and angiogenesis (Hanai et al. 2006; Uzzo et al. 2006; Makhov et al. 2008). This could potentially be a mechanism of decreased cell proliferation at the tissue level although the significance of this finding in a cell culture (which does not depend upon the formation of new vessels) is unclear.

One might expect an opposite effect of zinc on the transcription levels of caspase-3 or VEGF as compared to TPEN, since TPEN is a chelator of zinc. However, this was not the case in this research. One possible reason is that the doses used were not equivalent. The other possible reason is that TPEN has other effects besides zinc chelation. This could be chelation of copper in addition to zinc (Makhov et al. 2008), which could explain its role in decreasing VEGF.

Significance of the finding and future research

Previous research has shown that colorectal cancer cells metastasised to the liver might be trying to retain zinc in the cells, although the mechanism is poorly understood (submitted for publication). This research has shown that chelation of zinc from HT-29 colorectal cancer cells results in cell death. Further

research by repeating the research in different cell lines, use of micro-array methods to determine the transcription levels of the entire transcriptome, and the use of more frequent time intervals may lead to better understanding of the mechanism and importance of zinc transport in cancer cells and may result in novel therapeutic options.

Conclusions

Zinc chelation of HT-29 cells causes cell death. Zinc appears to be protective for short periods of exposure to TPEN but has no protective effect on prolonged exposure. HT-29 cells are not able to counteract the effect of intracellular chelation of zinc by altering zinc transport. Further research into the mechanisms of these findings is necessary and may lead to novel therapeutic options.

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