The Effect of Se-Methylselenocysteine on Human Transcription Factor E2F1 Expression Levels in HeLa Cells

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Abstract:

Selenium is a trace element essential to the mammalian diet, (Yin *et al.* 2004) and has been shown to inhibit the growth of mammalian cells (Sinha and Medina), suggesting a role in cancer treatment. E2F is a family of transcription factors that control transcription of genes required for cell cycle progression (Goodwin and DiMaio 2000), and its inhibition inhibits progression of the cell cycle (Lodish 2003). E2F1, an E2F family member that stimulates transcription, is specifically responsible for activating genes that are required for G1/S-phase transition and cell cycle progression during the S-phase of the cell cycle. Mutations in pRB and other molecules regulating the pRB/E2F1 interactions are often mutated in human cancers, resulting in deregulated activity of E2F1 (Ginsberg 2002). The ability to decrease the level of E2F1 expression, would result in the loss of uncontrolled cell growth in cancerous populations and have an antiproliferative effect on cancerous tumors. To determine whether selenium treatment impacts E2F1 in a cervical cancer cell line, HeLa cells were treated in the absence or presence of Se-methylselenocysteine. Primers specific for E2F1 were created and used to isolate and amplify E2F1 RNA in an RT-PCR reaction. The products were run on an agarose gel and the amount of product present was quantified and statistically analyzed to determine whether E2F1 RNA levels were affected by treatment with selenium. No difference in RNA expression levels was observed.

Introduction:

Selenium is a trace element essential to the mammalian diet, (Yin *et al.* 2004) and has been shown to inhibit the growth of mammalian cells (Sinha and Medina), suggesting a role in cancer treatment. A 2005 study on the effect of selenium treatment on skin cell carcinomas revealed that selenium has an antiproliferative effect on three types of cancers: cancers of the lung, prostate and breast (Clark *et al* 2005). More recent studies have suggested that at levels exceeding the nutritional requirement (Ganther 1999), selenium will also reduce the risk of cancer in the human stomach, mouth (Brash and Havre 2002) and colon (Yin *et al.* 2004).

Many forms of selenium exist. Methylated forms of selenium such as methylselenocysteine (MSC) and methylselenic acid (MSA) are less toxic than non-methylated varieties (Ganther 1999, Ip *et al.* 1998). MSC, a non-methylated organic selenium compound (Sinha and Medina), is a potentially powerful agent in human cancer treatment. MSC has been studied in clinical trials with prostate, lung, and colon carcinoma (Yin *et al.* 2004) and is suggested to be the most effective selenium treatment for breast cancer (Unni *et al*. 2005).

E2F is a family of transcription factors that control transcription of genes required for cell cycle progression (Goodwin and DiMaio 2000), and its inhibition will therefore inhibits progression of the cell cycle (Lodish 2003). E2F1, an E2F family member that stimulates transcription, is specifically responsible for activating genes that are required for G1/S-phase transition and cell cycle progression during the S-phase of the cell cycle (Quin *et al*. 1994, Ginsberg *et al*. 2002). E2F1 is bound to and controlled by pRB, which represses E2F activity during quiescent periods. In order for replication to occur, a cyclin/cdk complex phosphorylates pRB to inactivate it, allowing E2F to induce transcription (Dick and Dyson 2003). Mutations in pRB and other molecules regulating the pRB/E2F1 interactions are often present in human cancers, resulting in deregulated activity of E2F1 (Ginsberg 2002). Approaches that would enable a decrease the level of E2F1 expression should result in the loss of uncontrolled cell growth in cancerous populations and have an antiproliferative effect on cancerous tumors.

The purpose of this study was to elucidate whether the addition of selenium to cervical cancer cells would have an inhibitory effect on E2F1 expression levels. Cervical cancer, a disease with which about 14,000 women are diagnosed every year (NCCC

2006), is of unknown cause. HeLa cells are a cervical cancer cell line expressing E2F, making them a suitable cell line in which to examine the impact of selenium treatment.

Materials and Methods

Cell Treatment: HeLa cells were cultured in MEM with 10% feral calf sera, 10 μg/ml streptomycin and10 IU/ml penicillin and incubated at 37°C with 5% CO_2 and humidity. The cells were then divided into three groups and treated with Se-methylselenocysteine (solubilized in water at a concentration of 50 mg/ml) at concentrations of 60 μmol. and 120 μmol.

Primer design: Primers were designed to selectively amplify E2F1 mRNA. Sequences with low homology to other genes and to E2F1 homologs 2-8 were chosen. Primers were designed to span an intron and to amplify 284 bases of mRNA. Should genomic DNA be present, a PCR product of greater than 1500 bases would be generated. The primers employed to amplify the E2F1 mRNA were as follows: E2F1 (NM_005225.1) forward sequence: exon 3, GGAGAAGTCACGCTATGAGACC, bases 492-513; reverse sequence: exon 4, GCTCGCTCTCCTGCAGCTGTC, bases 755-775. GAPDH (NM_002046) was used as a control (Zhao *et al*. 2003) : forward sequence AACGGATTTGGTCGTATTGG, bases 100-119; reverse sequence TTTGGAGGGATCTCGCTCCT, bases 308-327.

RNA isolation: Total RNA isolation was performed using the RNAqueous Kit from Ambion. RNA concentration was measured using a DU 530 Life Science UV/Vis spectrophotometer.

RT-PCR: The Qiagen One-Step RT-PCR kit was used according to the manufacturer's instructions. The E2F1 and GAPDH RT-PCR reactions were run in same thermal cycler. Each reaction contained 3 μL RT-PCR buffer, 0.6 μL dNTPs, .75 μL of each primer, 0.6 μL RT-PCR enzyme mix, RNA and RNase-free water to bring the final concentration up to 15 μL. RNA concentrations differed based on the reaction performed: 10 ng for E2F1 and 5 ng for GAPDH. 12 μL mineral oil was loaded on top of the reactions to prevent evaporation during thermocycling. Cycling conditions were as follows: 50°x 30 min and 95° x 15 min, followed by cycles of 94°x 20s, 59°x 30s, 72° x 30s. Cycle number varied by primer set: GAPDH was run for 25 cycles and E2F1 for 33 cycles. A final extension of 72° x 2min was carried out for all reactions.

Agarose Gel: Samples were run on a 1% agarose gel to confirm amplified sequence sizes and reveal any contamination or primer dimers.

PCR product purification: Purification to remove residual primers, dNTP, and Taq was carried out according to the manufacturer's instructions.

DNA Sequencing: Sequencing reaction by the Sanger di-deoxy method was performed with the AmpliCycle DNA Sequencing Kit. The sequencing reaction for each primer contained 4 μL 10x cycling buffer, 0.5 μL α ^{33}P, 50 fmol DNA, and enough ddH_2O to bring the volume up to 28 μL. Conditions were as follows: 3 minutes at 94 degrees, followed by 35 cycles of 30 seconds at 94 degrees, 30 seconds at 58 degrees, and 1' at 72 degrees. The samples were then cooled to 4 degrees, stop solution was added, and a

sequencing gel was run. The sequences obtained were then compared to the known E2F1 sequence from GenBank (NM_005225.1) in a BLAST search.

Results:

RT-PCR was performed on RNA isolated from HeLa cells incubated under three sets of conditions: treatment with 60 µmol. Se-methylselenocysteine (MSC), 120 µmol. MSC and no MSC. The reaction was performed on each set of RNA with primers designed for E2F1 and GAPDH (control) so that any apparent differences in RNA expression could be normalized to GAPDH, which is unaffected by selenium (Zhao et al. 2005). The primer set for E2F1 amplified a product of around 300 base pairs, which matches the size of the E2F1 product that should be generated from the E2F1 transcript.

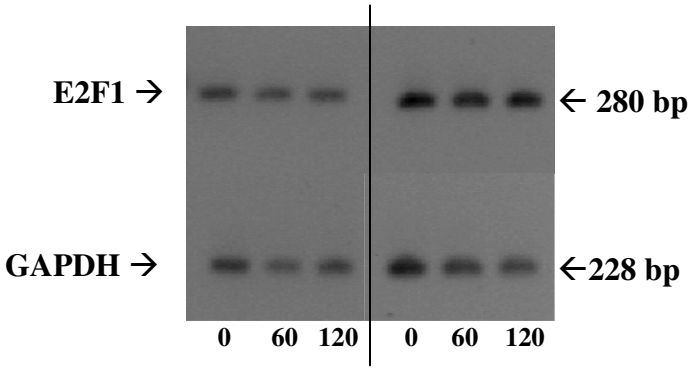

Figure 1. **RT-PCR analysis of E2F1 RNA levels as treated with 0 µmol., 60 µmol. and 120 µmol. Se-methylselenocysteine**. The above gel contains two replicates of the same PCR reaction. Lanes 1-3 show E2F1 and GAPDH (control) as treated with no MSC, 60µmol. MSC and 120 µmol. MSC respectively. Lanes 4-6 show a duplicate reaction of the same treatments. E2F1 primers (NM_005225.1) were located in exons 3 (492-513), and exon 4 (755-775) and designed to amplify a segment of 284 bases. E2F1 reactions were run for 33 cycles with 10ng RNA. GAPDH primers (NM_002046) were designed to amplify a segment of 228 bases. GAPDH reactions were run for 25 cycles with 5ng RNA.

To confirm that the primers designed for E2F1 were amplifying the correct mRNA segment, a sequencing reaction was run and the sequence generated was compared to E2F1 (NM_005225.1) using NCBI BLAST and determined to match the E2F1 mRNA sequence.

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mRNA    1    GACCTGAACTGGGCTGCCGAGGTGCTGAAGGTGCAGAAGCGGCGCATCTATGACATCACC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
E2F1    525  GACCTGAACTGGGCTGCCGAGGTGCTGAAGGTGCAGAAGCGGCGCATCTATGACATCACC  584

mRNA   61    AACGTTCCTTGAGGGCATCCAGCTCATTGCCAAGAAGTCCAAGAACCACATCCAGTGGCT  118
             ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
E2F1    585  AACGT-CCTTGAGGGCATCCAGCTCATTGCCAAGAAGTCCAAGAACCACATCCAGTGGCT  643

mRNA    119  GGGCAG  124
             ||||||
E2F1    644  GGGCAG  649
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Figure 2. **Sequence alignment of primer product and E2F1.** The mRNA product obtained with designed primers was sequenced and aligned with E2F1 (NM_005225.1) with a BLAST search.

RNA expression appears comparable in both replicates of all three treatment concentrations. To normalize for GAPDH amounts, QuantityOne software from BioRad was used to quantitate differences in RNA expression as related to GAPDH. The background was set , the number of pixels was determined and averaged for the two replicates of each sample.

Sample	Intensity 1	Intensity 2
Untreated E2F1	40.655	67.465
60 µmol. E2F1	31.312	54.151
120 µmol. E2F1	35.693	63.004
Untreated GAPDH	42.201	56.066
60 µmol. GAPDH	23.952	41.301
120 µmol. GAPDH	38.326	33.551

Figure 3. **Quantitation of RNA expression**. RNA expression of E2F1 normalized to GAPDH (control) levels was obtained using Quantity One software from BioRad.

E2F1 band intensities were normalized to GAPDH amounts, and an ANOVA analysis was then performed to determine whether differences in E2F1 RNA amounts could be attributed to expression levels. Calculations showed that the null hypothesis could not be rejected, indicating that the variance among the E2F1 selenium-treated samples was not significantly different.

H_0: variance among samples = variance within samples
H_1: variance among samples ≠ variance within samples

	Untreated	60µol. MSC	120 µmol. MSC	Total
Reaction 1	.963	1.307	.931	
Reaction 2	1.203	1.312	1.878	
ΣY	2.166	2.619	2.809	7.594
Mean	1.083	1.310	1.405	
ΣY^2	2.375	3.430	4.394	10.199
Total	4.692	6.860	7.890	19.442

Correcting term: 9.611
Total sum of squares: 0.588
Sum of squares among: 0.109
Within sum of squares: 0.479

Variance	Df	SS	MS
Among	2	0.109	0.0545
Within	3	0.479	0.160
Total	5	0.588	

f_{calc}= 0.340625 $f_{statistic}$= 0.4775

Figure 4. **Three-way ANOVA analysis of E2F1 expression levels as compared to GAPDH levels.** Statistical analysis to determine whether apparent differences in RNA levels were due to a difference in expression level or were not statistically significant. Since f_{calc} was less than $f_{statistic}$ the null hypothesis was not rejected, and no difference in RNA expression could be shown.

Discussion:

In this study, total RNA was isolated from HeLa cells having been incubated with selenium for 24 hours, and RT-PCR reactions were run with primers were designed to amplify E2F1 in HeLa cells. After confirming that the product generated by primers designed to amplify E2F1 was correct, analysis of RNA expression levels of E2F1 was performed. QuantityOne software was used to quantitate apparent differences in RNA expression, and an ANOVA was computed to determine the relevance of the RNA levels. Based on this information, Se-Methylselenocysteine was determined to have no effect on the level of E2F1 expression in HeLa cells.

Given the evidence provided by many authors that selenium has an inhibitory effect on E2F gene expression in many cancer cell lines, it would seem reasonable to expect that inhibition by selenium would also occur in cervical cancer. Since no information on HeLa cell treatment with selenium was available, two different dosage levels were used for a period of 24-hours, in hopes that one of these dosages would have an inhibitory effect on RNA expression. Dosage levels were selected based on previous studies with similar, but not identical, experimental procedures (El-Bayoumy and Sinha 2005, Ip 1998, Unni *et al*. 2005, Venkateswaran *et al*. 2002). The amount of selenium and/or duration of treatment with MSC appear to affect the level of inhibition of E2F1 expression (Unni 2005), and it is possible that the concentration of selenium or the incubation interval used in this study were not sufficient to impact the expression of E2F1.

It is also possible that the type of selenium used did not have an optimal effect on the cell type. MSC is an effective treatment for breast cancer cells (Unni et al. 2002) while MSA is most effective in prostate cells (El-Bayoumy and Sinha 2005). Further

testing on HeLa cells with MSA or another form of selenium other than MSC might have revealed an inhibition of E2F1 expression.

Although no difference in E2F1 expression levels was observed in HeLa cells, it is still possible that the addition of selenium to the human diet could suppress cervical cancer growth. A 2002 study on mouse mammary cells by Dong et al. showed that, while the impact of various dosages of various selenium treatments varied greatly in vitro, their in vivo effect was the same. It has been suggested that higher dietary intake of selenium in animals corresponds with lower overall cancer rates (Ip *et al*. 1998). A four-and-a-half year study to determine the effect of dietary selenium on skin cell carcinomas revealed that patients receiving the selenium supplement (200 μg/day, roughly twice the average daily intake) did not have lower rates of skin carcinomas, but did have much lower frequencies of lung, colon and prostate cancers (Clark *et al*. 1996). Since the participants were almost exclusively men, no information could be gathered on the effect of dietary selenium on cervical cancer prevalence.

Possible future studies include increasing the amount and/or duration of selenium used on HeLa cells and incubating cells with alternate forms of selenium. Any information gained from these studies will be helpful in revealing the impact of selenium on cancer cells and the possible uses for selenium in cancer prevention and treatment.

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