Expression and CpG Methylation of the Insulin-like Growth Factor II Gene in Human Smooth Muscle Tumors

Ton Gloudemans, Ina Pospiech, Leo T. M. Van Der Ven, Cornelis J. M. Lips, Hélène Schneid, Willem Den Otter, and John S. Sussenbach

ABSTRACT

Previously we have shown that expression of the insulin-like growth factor II (IGF-II) gene in 36 normal smooth muscle tissues (myometria) and 26 benign smooth muscle tumors (leiomyomas) was detectable by Northern blot analysis but that the RNA levels were low. In 9 of 20 malignant smooth muscle tumors (leiomyosarcomas) IGF-II gene expression was also low or absent, while in 11 of 20 the IGF-II gene was abundantly expressed.

In 32 of these tissues we have now studied the DNA methylation state of the IGF-II gene. For the analysis of overall methylation of the gene the restriction endonucleases HpaII and MspI were used. In normal smooth muscle and in leiomyomas the IGF-II gene appeared to be methylated. In leiomyosarcomas with low IGF-II gene expression the DNA was partly demethylated. In leiomyosarcomas with abundant IGF-II gene expression overall methylation of the DNA tended to be low. In addition, we have studied the methylation state of one particular CpG site in the IGF-II gene with the restriction endonuclease AvaII. The results of the latter analysis confirm the analysis with HpaII and MspI.

In conclusion, in malignant smooth muscle tumors the data indicate an inverse correlation between CpG methylation and expression of the IGF-II gene.

INTRODUCTION

Human IGF-II is a polypeptide of 67 amino acids, structurally homologous to insulin and IGF-I. The main production site for IGF-II is fetal liver and, at a lower level, it is also produced in adult liver and many other tissues. The polypeptide has growth-promoting and insulin-like activities (1, 2). In addition to an endocrine mode of action of IGF-II, local effects have also been involved in paracrine or autocrine growth regulation.

The genetic organization of the IGF-II gene is complex. The human gene consists of at least 9 exons and has 4 promoters, designated P1–P4, which are activated in a developmental and tissue-specific manner (5–8). Promoter P1, located in front of exon 1, is active only in adult liver tissue, while promoters P2, P3, and P4 are quite active in fetal tissues and at a low level in several adult tissues. Fig. 1 depicts the IGF-II gene with the location of the 4 promoters and the different mRNAs transcribed from this gene. Despite the differences in the 5’ and 3’ untranslated regions of the IGF-II messengers, they all code for the same form of mature IGF-II peptide.

IGF-II may play an important role in the growth of some tumors. In several tumors large quantities of IGF-II have been found, and the IGF-II gene in many of these tumors is expressed, compared to normal tissue (3, 9). In addition, the type I IGF receptor, which mediates the growth-promoting effects of IGF-I and IGF-II (10, 11), is present in many IGF-II-overexpressing tumors (3, 9). Previously, we have shown that in 55% of the malignant smooth muscle tumors (leiomyosarcomas) the IGF-II gene is highly expressed compared to normal smooth muscle tissue or benign smooth muscle tumors (leiomyomas) (12). This suggests a role for IGF-II in the growth of malignant smooth muscle tumors.

The enhanced expression of the IGF-II gene in tumors might be due to modifications in the structure of the gene, like rearrangements, amplifications, or point mutations. In one case of a colon adenocarcinoma a structural aberration in the IGF-II gene region was reported (13) as a rearrangement located just outside the IGF-II gene, 3’ of exon 9. Another case of an altered IGF-II gene has been found in a Wilms’ tumor, where the gene showed a rearrangement (14). No other report provides evidence for gross gene rearrangements or amplifications (15–18). Other causes for the high IGF-II gene expression in some tumors might be the altered composition of the transcription factors in malignant tumors or epigenetic modification of the IGF-II gene (e.g., methylation).

There is much evidence now that methylation of CpG base pairs in DNA plays a role in eukaryotic gene regulation (19). A correlation between methylation state and activity of specific genes has been demonstrated. Experiments with genes that have a tissue-specific expression pattern have shown that in tissues in which the gene is expressed the gene is demethylated, while in tissues with low or no expression the same gene is methylated (20). When some specific genes were methylated in vitro and introduced into cells, the activity of these genes was repressed, compared to the unmethylated gene (21, 22). These data suggest that changes in methylation state may modulate gene activity.

In several types of tumors the extent of DNA methylation of the IGF genes was highly variable, as was the level of IGF gene expression (23). The goal of this study was to find the cause of the high expression of the IGF-II gene in many human leiomyosarcomas. Therefore, in normal smooth muscle, leiomyomas, and leiomyosarcomas the sizes and the levels of IGF-II mRNAs were studied. By Southern blot analysis it was determined whether rearrangements or amplifications had occurred in the IGF-II gene. The level of overall DNA methylation of the IGF-II gene was determined by analyzing the DNA with methylation-sensitive restriction endonucleases. In addition, quantification of the methylation level was performed by studying the methylation state of one particular CpG base pair in the IGF-II gene.

MATERIALS AND METHODS

Tissues. Tissues were obtained after surgery. Normal uteri were from women undergoing hysterectomy, leiomyomas were obtained from women undergoing hysterectomy, and leiomyosarcomas were obtained from women undergoing surgical resection.
After gentle mixing, sodium dodecyl sulfate was added at a final concentration of 1%. The suspension was gently mixed for 14 h at 37°C for protein digestion. The genomic DNA was purified by repeated phenol-chloroform extraction. Large-molecular-weight DNA was isolated by precipitation with 1 volume isopropanol and winding of the precipitate around a glass rod. The DNA was dissolved at room temperature in 10 mM Tris-HCl-1 mM EDTA, pH 8.0, for 14 h. DNA concentrations were measured using a fluorometric assay with the dye Hoechst 33258.

Genomic DNA was digested with several restriction endonucleases. For detection of gene rearrangement or amplification EcoRI, HindIII, and SaI1 (Boehringer Mannheim, Germany) were used. HpaI, Mspl (Boehringer Mannheim, Germany), or AvaII (New England Biolabs, MA) was used to determine DNA methylation. Typically 10 µg DNA were digested first with 30 units restriction endonuclease for 2 h; then 50 units restriction endonuclease were added for an additional 14-h digestion. Digestion was monitored by checking for complete digestion of phage λ DNA added to an aliquot of the digestion mix.

Equal amounts of the digested DNA were size-separated by electrophoresis in 1% agarose gels and transferred to GeneScreen (New England Nuclear) membranes. After baking at 80°C for 2 h the blots were prehybridized for 6 h at 42°C in 50% (v/v) deionized formamide-1 M NaCl-50 mM Tris-HCl (pH 7.5)-0.2% bovine serum albumin (fraction V)-0.2% polyvinylpyrrolidone-0.2% Ficoll 400-0.1% sodium pyrophosphate-1% sodium dodecyl sulfate-10% dextran sulfate-100 µg/ml denatured herring sperm DNA. Hybridization was performed in the same solution supplemented with a denatured 32P-labeled complementary DNA probe (0.5–1 ng/ml) for 20 h at 42°C. Blots were washed to a final stringency of 15 mM NaCl-1.5 mM sodium citrate-1% sodium dodecyl sulfate at 65°C for 30 min, and exposed to prefished Fuji RX film with two intensifying screens.

Hybridization Probe. The 713-base pair PstI insert of plasmid IGF-IIvar (28) was used as a complementary DNA probe for the IGF-II gene and mRNA. This probe contains nucleotide sequences derived from exons 3, 7, 8, and 9 of the human IGF-II gene (Fig. 2, A and B). This DNA was labeled with [α-32P]dCTP by the method of random priming (29) to a specific activity of 0.5–2 × 10^8 cpm/µg DNA.

RESULTS

Sizes and Quantities of IGF-II mRNA. Total RNA was isolated from 36 normal uterus myometria, 26 leiomyomas from the uterus, and 20 leiomyosarcomas from other origins. Northern blots of these RNAs were hybridized with the IGF-II-specific complementary DNA probe.

In all smooth muscle tissues the same IGF-II mRNA species are found as detected in human fetal liver (12). The major IGF-II mRNAs are a 6.0-kilobase and a 2.2-kilobase species, transcribed from promoter P3; a 4.8-kilobase mRNA, transcribed from P4; and a 5.0-kilobase mRNA, transcribed from P2 (Fig. 1). The 5.0-kilobase mRNA migrates at approximately the same position as the 4.8-kilobase mRNA and is, therefore, often not distinguishable. In normal myometria and in leiomyomas low quantities of 4.8/5.0- and 6.0-kilobase mRNAs were found. As described before (12), in 9 leiomyosarcomas no IGF-II mRNA was detectable or the level was very low. In the other 11 leiomyosarcomas, however, expression of the IGF-II gene was abundant. Laser densitometric scanning of the fluorographs revealed that the level of IGF-II mRNAs in these leiomyosarcomas is at least 30-fold higher than in normal.
smooth muscle. In Fig. 3 some examples are shown; in Fig. 3A the low level of IGF-II gene expression in normal smooth muscle and leiomyomas is compared with the high level in human fetal liver. Fig. 3B shows leiomyosarcomas with high IGF-II gene expression and leiomyosarcomas in which expression of the IGF-II gene is undetectable.

In summary, in smooth muscle tissue the same IGF-II gene transcripts are found as in fetal liver, and in 55% of the malignant smooth muscle tumors IGF-II gene expression is enhanced by 30-fold or more as compared to normal smooth muscle tissue.

**CpG Methylation State of the IGF-II Gene.** Alterations in the structure of the IGF-II gene might cause overexpression of this gene in 55% of the leiomyosarcomas. However, Southern blot analysis with the restriction endonucleases EcoRI, HindIII, and SalI provided no evidence for gross gene rearrangement or amplification in any of the smooth muscle tissues or tumors (data not shown). An other explanation for the high expression of the IGF-II gene might be epigenetic modification of this gene. We studied, therefore, the state of methylation of CpG base pairs in several smooth muscle tissues and tumors.

Genomic DNA was isolated from 9 normal uterus myometria, 13 leiomyomas, and 5 leiomyosarcomas with low IGF-II gene expression. In addition, genomic DNA was isolated from 5 leiomyosarcomas in which the IGF-II gene was highly expressed. The DNA was digested with either of the restriction endonucleases MspI or HpaII. Both restriction endonucleases cleave the sequence 5'-CCGG-3', but the activity of HpaII is inhibited when in the internal CpG base pair the C-residue is methylated. MspI, on the other hand, is not inhibited by methylation of this C-residue (30).

In Fig. 2A the location of MspI recognition sites in and around exons 3, 7, 8, and 9 of the IGF-II gene is shown. The expected sizes of the restriction fragments hybridizing with the IGF-II complementary DNA probe are shown in Fig. 2C. Since intron sequences are not fully known, the sizes of two of the hybridizing fragments cannot be determined by sequence.

Digesting genomic DNA with MspI reveals hybridizing fragments of 1170, 350, and 230 base pairs, as shown in Fig. 4. These fragments are derived from exons 7, 8, and 3, respectively (Fig. 2C). Furthermore, a RFLP is observed of 1000 and 850 base pairs. This RFLP was described before (31, 32). After the digestion of genomic myometrium DNA and leiomyoma DNA with HpaII, the fragments that were detected with MspI were absent. However, in the HpaII-digested DNAs larger-size hybridizing fragments were found (Fig. 4). This indicates that in these tissues many CpG base pairs in the IGF-II gene are methylated. Addition of increasing amounts of MspI, up to a 32-fold excess, did not alter this pattern (data not shown). In the 5 leiomyosarcomas with absent or low IGF-II gene expression, larger-size hybridizing fragments are also found with HpaII. In 4 of these tumors fragments of the sizes detected with MspI were also present. In Fig. 4 three examples are shown (LMS 9, LMS 10, and LMS 3).

In the 5 leiomyosarcomas with very high IGF-II gene expression the demethylation is almost complete. Fig. 4 shows one example (LMS 1). Table 1 summarizes the results of the overall methylation analysis. It is evident that in normal tissue and benign tumors the IGF-II gene is invariably methylated, whereas in the malignant tumors the gene is partially demethylated. The demethylation is almost complete in the malignant tumors which have a high IGF-II gene expression.

**Quantification of CpG Methylation State.** By using the restriction endonuclease pair MspI/HpaII the overall methylation state of the IGF-II gene can be assessed. However, with this method it is not possible to obtain quantitative data on the methylation state. Therefore, we used the restriction endonuclease AvaII, which cleaves the sequence 5'-GG(A/T)CC-3', but not 5'-GG(A/T)Cm5C-3' (m5C = 5-methyl-cytosine) (23). Fig. 2A depicts the AvaII sites in and around exons 3, 7, 8, and 9. Since in mammals a C-residue is only methylated when it is present in the context CpG (33), only the AvaII site within exon 7 is methylation-sensitive because it is only there that the sequence 5'-GGACCG-3' is found. Digestion of genomic DNA with AvaII results in a fragment of 1620 base pairs when this particular site is methylated and in 620- and 1000-base pair fragments when it is not methylated (Fig. 2D). Determination of the ratio of the amounts of the 620- and 1620-base pair fragments allows quantification of the methylation state of this particular CpG base pair in the IGF-II gene (23).
Expression and Methylation of the IGF-II Gene

Genomic DNA isolated from the previously mentioned tissues and tumors was digested with AvalII and subjected to Southern blot analysis. Hybridization with the IGF-II complementary DNA probe also revealed, besides the described fragments of 1620 and 620 base pairs, hybridizing fragments of 1100 and 900 base pairs. The latter two fragments were described previously as a RFLP (34), and the polymorphic site is located in exon 9 (data not shown). The IGF-II complementary DNA probe we have used also contains exon 3 sequences which hybridize to a 900-base pair fragment migrating at exactly the same position as the 900-base pair RFLP fragment of exon 9 (data not shown). A fragment of 400 base pairs was also found which is from exon 8. Fig. 5 shows some typical results. The appearance of the 620-base pair fragment in genomic DNA from tissues overexpressing the IGF-II gene is evident.

The fragments were quantified by laser densitometric scanning of the fluorographs. The methylation of this AvalII site was determined by the ratio of the 620-base pair fragment intensity to that of the sum of the 620-base pair and the 1620-base pair fragments, as described (23). The intensity of the other fragments was regarded as an internal control. The results are summarized in Table 1. A strict inverse correlation between IGF-II gene expression and the methylation of this particular CpG base pair is observed.

Discussion

We have shown an inverse correlation between expression of the IGF-II gene and the overall methylation level of this gene in human smooth muscle tissue and tumors. In normal smooth muscle and benign smooth muscle tumors expression of the IGF-II gene is low or absent. In all these tissues the IGF-II gene appeared to be methylated at a high level. In the leiomyosarcomas in which we detected low or absent IGF-II gene expression, the overall methylation state varied from methylated to partly demethylated. In the leiomyosarcomas with high IGF-II gene expression the gene was always demethylated. By studying one particular CpG base pair within exon 7 of the gene we were able to quantify the level of methylation. In this case a strong inverse correlation between IGF-II gene expression and methylation was found. These results are in agreement with the overall methylation data of the IGF-II gene.

Table 1 Correlation between the presence of IGF-II mRNA and the methylation state of the IGF-II gene

<table>
<thead>
<tr>
<th>Tissue</th>
<th>IGF-II mRNA Level</th>
<th>Methylation of the IGF-II gene</th>
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<td></td>
<td></td>
<td>HpaII/Msp I (overall)</td>
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<tr>
<td>Normal uterus</td>
<td>Low</td>
<td>High</td>
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<tr>
<td>Leiomyoma</td>
<td>Low</td>
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<td>Leiomyosarcoma</td>
<td>Low</td>
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<td>Normal uterus</td>
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The level of methylation of the whole genome in malignant tumors and metastases is often lower than the level in benign tumors and healthy tissue (35). This suggests that during tumor progression overall demethylation of the genome frequently occurs. On the other hand, in leukemia and small cell lung carcinoma most genes on the short arm of chromosome 11 are methylated (36, 37). This methylation might result in the loss of function of several genes in this part of the chromosome. This does not hold for leiomyosarcomas since (a) demethylation of the IGF-II gene is not a general phenomenon in these tumors and (b) the IGF-II gene in leiomyosarcomas does not become methylated. In normal smooth muscle and leiomyomas the IGF-II gene is already methylated and in only 55% of the leiomyosarcomas is the gene demethylated. Therefore, a more specific mechanism for demethylation of the IGF-II gene is likely. It cannot be excluded that in the 5 leiomyosarcomas in which the IGF-II gene is demethylated other genes on the short arm of chromosome 11 are also demethylated. The inverse correlation between methylation and IGF-II gene expression could be specific for certain tissues. We have observed this inverse correlation for smooth muscle tissue and its tumors, and Schneid et al. (23) have found it for some other tissues.

When we studied one particular CpG base pair in exon 7 the levels of IGF-II gene methylation varied from 85 to 100% in normal smooth muscle tissue. Demethylation of the IGF-II gene in only a few cells in these tissues can explain the low level of expression of the IGF-II gene. We could not find a correlation between variations in the level of methylation and the small changes in IGF-II gene expression level in these tissues. Furthermore, in tissues with a methylation level up to 100%, the IGF-II gene is still expressed. Therefore, in normal smooth muscle tissue a basal level of IGF-II gene expression persists, irrespective of the methylation state of the gene. The same low level of IGF-II gene expression was also observed in other cases where the IGF-II gene is methylated (23).

In benign smooth muscle tumors the level of IGF-II gene methylation is the same as in normal smooth muscle tissue. Expression of the IGF-II gene, however, is slightly enhanced in these tumors (12). This might reflect either a general gene activation or the fact that in some cells the IGF-II gene is highly expressed but in most cells it is not. In the latter case a change in DNA methylation state in leiomyomas, compared to normal tissue, is expected. The fact that we cannot demonstrate this change might be due to insufficient sensitivity of our method. General activation of the IGF-II gene irrespective of methylation level, however, cannot be excluded. A difference in promoter use between tissues that express the IGF-II gene at low or high levels has been described (12). Normal smooth muscle expresses the IGF-II gene at low levels, and promoter P4 is more active than P3, while in leiomyosarcomas that express the IGF-II gene at high levels P3 becomes more active than P4. In leiomyomas, which all have a low level of IGF-II gene expression, a shift in promoter use from P4 to P3 was already observed (12).

Fig. 5. Southern blot hybridized with 32P-labeled IGF-II complementary DNA probe. DNA digested with AvalII from two normal myometria (UT 1, UT 2), one leiomyoma (LM 3), and six leiomyosarcomas (LMS 2–5, LMS 7, LMS 8). Left ordinate, sizes of fragments in kilobases. Abscissa, level of IGF-II gene expression in these tissues and tumors: +, high expression; −, low expression. Bottom, percentage methylation as determined by laser densitometric scanning.
The higher expression of the IGF-II gene in leiomyomas, therefore, probably is a consequence of high expression of the IGF-II gene in only some cells in this tissue. In situ hybridization of IGF-II mRNA may give additional information about which cells express the IGF-II gene in normal smooth muscle and in leiomyomas.

In leiomyosarcomas we can distinguish two types with either high or low IGF-II gene expression (12). In the latter tumors IGF-II is not involved in growth or maintenance of the tumor state, but in the case of high IGF-II gene expression there might be a role for IGF-II in the growth of these tumors. It is conceivable that, due to demethylation, the IGF-II gene becomes highly expressed, resulting in high levels of IGF-II peptide. This IGF-II serves as an autocrine or paracrine growth factor for these tumor cells. In rat uterus the type I IGF receptor, which can bind to IGF-II, is predominantly present on the smooth muscle cells (38); so overexpression of the IGF-II gene might lead to growth stimulation via this receptor.

Regions of interest for CpG methylation are the CpG islands in promoters (39). In the human IGF-II gene promoters P3 and P4 have a high CpG content (40). These promoters give rise to mRNAs of 6.0 and 4.8 kilobases, which are the mRNAs predominantly found in tumors overexpressing the IGF-II gene. Our data clearly show an inverse correlation between high IGF-II gene expression and overall demethylation of this gene. Whether this is also reflected in demethylation of the promoter regions is not known. A possible cause for the overall demethylation is aberrant activity of a maintenance CpG methylase.

During DNA replication, the maintenance CpG methylase recognizes the hemimethylated DNA and methylates the newly formed strand (41, 42). If this enzyme is affected or inhibited, demethylated DNA will result and possibly genes are expressed that should not be activated (43). It was shown recently that the inhibition of transcription by methylation is an indirect effect, involving a methyl-binding protein (44), and this protein or its gene might also be affected. Inhibition of the methylase or the methyl-binding protein will result in a general demethylation of the genome. In leiomyosarcomas the genome is not generally demethylated (23). More likely, a change in the IGF-II gene or in the chromosomal region where the gene is located (11p15) is the cause for the demethylation. Rearrangements of the IGF-II gene, however, were not detected by us, and in one case a point mutation can also be excluded (45). Outside the IGF-II gene, DNA integrity was not studied by us. In several cases of Wilms' tumor, in which the IGF-II gene often is expressed at a high level, chromosomal aberrations in the 11p15 region have been reported (46-48).

In conclusion, we have shown a correlation between high IGF-II gene expression and DNA demethylation in leiomyosarcoma. This aberrant demethylation might be the cause of high IGF-II gene expression and high IGF-II peptide production. The IGF-II peptide then can act as an autocrine or paracrine growth factor for these leiomyosarcoma cells because these cells also possess the type I IGF receptor. In normal smooth muscle tissue IGF-II gene expression is independent of the methylation state of the gene.

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