5 Discussion

5.1 MUC2 promoter sequence analysis

For the analysis of mechanisms of MUC2 expression regulation the promoter sequence was recovered from the genomic library. The obtained clones contained about 9 kb of the promoter sequence. However only 500 bp of the promoter sequence as well as 400 bp of transcribed sequence were analysed in detail. At the same time analysis of about 2.5kb of the promoter sequence of MUC2 gene was published by Gum et al. [101] and 5 kb were analysed by Velcich et al. [102]. The comparison of obtained and published sequences showed the 100% homology.

The computer analysis of the obtained promoter sequence revealed several transcription factor binding sites. Most of the detected elements, such as TATA box and Sp1 binding site are typically present in eukaryotic promoters. However, the comparison of computer detected transcription factor binding sites with experimentally analysed by Gum et al. [101] showed that only CACCC box has a strong influence on the promoter activity, whereas the others play only a minor role.

Another interesting element in the promoter sequence of MUC2 is the ATCC microsatellite located about 5 kb upstream the transcription start site. Being usually irrelevant to the promoter activity this microsatellite can serve as a good marker for the analysis of the allelic distribution of MUC2 gene in the population.

Analysed transcribed sequence revealed the size of the first exon – 102 bp what corroborates the published data [101,102]. The multiple exon structure and relatively big sizes of the introns [101] does not exclude the presence of enhancers downstream from the transcription start.

5.2 Regulation of MUC2 expression in cell lines.

In the present work the MUC2 promoter activity in reporter system did not correlate with the endogenous expression of MUC2 gene (Results 4.1.3). Gum et al. analysed the expression of MUC2 in strongly expressing cell line C1a in comparison with the nonmucinous and low expressing cell line HT-1080 [101]. The activity of the reporter in these cell lines differs only by the factor of 3, contradicting the results obtained in Northern blot. Velcich et al. showed further, that MUC2 promoter is strongly active in cervical carcinoma cell line HeLa, which does not express endogenous MUC2 [108]. The results obtained in current work are in agreement with these data, showing that the difference of the promoter activity determined in the reporter system in cell lines HT-29 and LS174T is about 2 fold, while the difference in the endogenous MUC2 expression is more than 10 fold. These results suggest the presence of an additional mechanism of regulation of the tissue specific expression of MUC2 gene.

Therefore the attempt of identification of tissue-specific enhancer for MUC2 gene was undertaken. However in the first and the second exons of MUC2 no enhancers were found. The sequences upstream of the gene were analysed for the promoter activity by Velcich et al. and there was no additional transcription activating activity observed [108]. Therefore if there is an enhancer, it can be located only upstream of –5000 bp or downstream of the third exon i.e. about 4 kb downstream.

Methylation of the promoter region of MUC2 gene was analysed in cell lines with different level of MUC2 expression. In cell lines with low expression of MUC2 the promoter region was almost completely methylated, confirming the results of Southern blot analysis. Similar correlation between the high level of methylation and suppression of calcitonin gene in colon cancer cell lines was observed [109].

The paradigm of the methylation-dependent expression of gene includes strong correlation of methylation and promoter activity, and the possibility to reactivate the gene, suppressed by methylation by means of the inhibition of methylation. This was indeed observed for hMLH1 and p16 genes, which are activated by the inhibition of methylation with 5-aza-2'-deoxycytidine [85,110]. Similarly, the treatment of low expressing cell line T84 increased the level of the expression of MUC2 gene 3 fold [96]. Additional experiments with cell line COLO 205 in which MUC2 expression is not detectable showed, that treatment of this cell line with 5-aza-2'-deoxycytidine induced *de novo* expression of MUC2.

The consistent correlation between low/high methylation of the MUC2 promoter region and high/low MUC2 expression in cell lines as well as the effect of 5-aza-2'-deoxycytidine strongly supported the hypothesis that methylation is regulating MUC2 expression.

If methylation is considered as an event preceding the changes of the cell phenotype, then MUC2 promoter methylation can be considered as an epigenetic marker of the mucinous pathway of the development of colon carcinoma.

However the experimental model used in these series is too complex due to the fact that most of the cell lines are polyclonal. Therefore cell line treated with 5-aza-2'-deoxycytidine was cloned. In this case clones with homogenous properties and methylation should be obtained. The obtained clones showed different levels of MUC2 expression as well as different levels of MUC2 promoter methylation. The weak point of all methods based on the inhibition of methylation with 5-aza-2'-deoxycytidine treatment is unspecificity of methylation inhibition by 5-aza-2'-deoxycytidine. The drug binds

irreversibly to the maintenance methyltransferase (DNMT1) and inhibits its function [111], thus influencing all methylation-dependent genes. Among these genes there can be transcription factor genes which can directly influence the expression of MUC2 gene, in a methylation-independent manner. In the present work in three clones with different levels of MUC2 expression the expression of the reporter plasmid containing nonmethylated MUC2 promoter did not correlate with the endogenous expression of MUC2. This suggests that the transcription factors activated through 5-aza-2'-deoxycytidine treatment play a minor if any role in expression of MUC2. Therefore it can be concluded that MUC2 promoter methylation plays the main modulatory role in this model.

The promoter region described to contain all essential elements [101,102] was analysed. The overall low methylation within the promoter region coincides with the high level of the expression of MUC2 (Fig. 19, Table 1). However overall methylation level was described to play a role mainly in the regulation of the expression of the promoters containing CpG islands. No specific sites were identified also for p16 and calcitonin [86,89]. Since MUC2 promoter does not contain the CpG island, the site-specific methylation in the regulation of MUC2 expression was expected. Especially intriguing was the finding of AP2 binding sites within the promoter region. This transcription factor was shown to be methylation-dependent in its binding to DNA [112].

The analysis of methylation in the specific sites showed that some sites are less methylated in MUC2 expressing clones in comparison to nonexpressing ones. Out of total 3 sites with statistically significant lower methylation one was located immediately in front of the transcription start site and within the binding site for AP2. The binding of AP2 to the methylation site is less efficient than to nonmethylated one [112] and therefore

in this case methylation may play modulatory role in the regulation of the expression of MUC2.

To test if the statistical differences in the methylation of the clones have indeed a biological meaning, two sites were analysed by mutagenesis. Mutational analysis showed, that the contribution of the site located immediately upstream of the transcription start site is the most pronounced. In contrast, other site appears to be irrelevant to the regulation of the expression of MUC2.

Correspondence between MUC2 expression and overall low methylation level together with the site specific effect suggests the dual mechanism of MUC2 expression regulation. The overall methylation function probably involves the effects of chromatin condensing with the increase of methylation density. The effect of site-specific methylation most likely is based on the changes of the conditions of AP2 transcription factor binding.

5.3 Regulation of MUC2 expression in malignant and normal colonic tissues

The initial analysis of methylation in tissues was done by Southern blot hybridisation technique using genomic DNA isolated from pieces of tumours and normal tissues. This method was successfully used for analysis of methylation of the promoter regions of p15 in lymphomas [113], PTEN/MMAC1 in prostate carcinoma [114], pS2 in breast carcinoma [115]. The analysis of DNA-methylation in colon carcinoma tissue yielded unclear results. Methylation of MUC2 promoter region was heterogeneous and similar in all samples, normal or tumour (unpublished observations). This was interpreted as a result of tissue inhomogeneiety. For clear cut results of tissue analysis, enrichment of the samples with the cells of certain phenotype was therefore required. Therefore microdissection technique [116] was applied. This method has, however, some

disadvantages, for example the DNA isolated from such material is fragmented, and the amplification of the fragments longer than 400bp is usually impossible [117]. The use of microdissected material for the analysis of methylation has not been yet described.

Analysis of the methylation of the promoter region in colon cancer patients showed the same correspondence between methylation and the phenotype as in cell lines. Normal tissue which was represented by microdissected crypts showed average methylation of 80% which can result from the mixture of goblet and columnar cells present in a crypt [118].

Tumour samples were represented by cells with well defined mucinous or nonmucinous phenotype. In the case of nonmucinous tumours the methylation was as high as 97%, indicating that cell impurities, if present were minor. It can be assumed, therefore, that nonmucinous tumour cells have 100% methylation within the promoter region, and therefore completely suppressed expression of MUC2. In mucinous carcinomas the methylation of the promoter region was about 50%. If all cells supposed to express MUC2, then it can be hypothesized that one nonmethylated allele is enough for the expression of MUC2. One allele expression manner is a feature of imprinted genes [119], and the 11p15.5 is a well described imprinting locus [120], therefore it allows to hypothesize that MUC2 is an imprinted gene. The testing of this hypothesis was beyond the scope of this work.

These findings showed, that the methylation in tumours is similar to methylation in cell lines with the same phenotype. Therefore the question arose if the altered methylation in the MUC2 gene promoter is a tumorigenesis-associated process or just the event which normally determines the phenotype of the cells in the crypt. Contradictory results on the

importance of MUC2 expression level for the prognosis of colon carcinoma [17-19] suggest that methylation may be irrelevant for tumorigenesis and is just a mechanism which normally regulates MUC2 expression and in the case of colon carcinoma is superimposed on the tumour development. However some other differences between mucinous and nonmucinous phenotypes such as p53 and Ki-ras mutation rates [14,20,21] still suggests the different pathways of carcinogenesis in the case of mucinous and nonmucinous tumours. To answer this question it was important to understand in detail how methylation of MUC2 promoter is regulated in normal colonic cells.

Analysis of methylation in normal colonic epithelium was performed using the modified technique of bisulphite sequencing. Cells for the analysis were selected by the immunohistochemical staining of MUC2 and therefore relatively pure population of MUC2 expressing cell was analysed. The result obtained was in very good agreement with data obtained in tumour samples. The level of methylation in normal MUC2-expressing goblet cells appeared to be about 50%, what corresponds to one nonmethylated allele per cell. Also this result indirectly gives an information about the methylation of MUC2 promoter region in other cells in the crypt. Since goblet cells comprise about 40% of cells in the crypt, then one methylated allele per cell (50% methylation) in this subpopulation of cells will result in 80% methylation when analysing the total crypt, the result which was obtained in this work. Therefore it is logical to suppose, that only goblet cells in the crypt contain one nonmethylated allele of MUC2 in their genome, and all other cells have completely methylated MUC2 promoter. These results also give a clue to understanding of the role of methylation in the development of normal colon epithelial cells. Goblet and columnar cells originate from the same stem cells which are MUC2 negative [121]. Therefore there must be a differentiation

mechanism which decreases methylation in goblet cells. Decrease of methylation can follow either by active demethylation or methylation inhibition during DNA replication. However reporter analysis showed that nonmucinous cell lines may have a high amount of transcription factors required for MUC2 expression and at the same time have heavily methylated MUC2 promoter. This indicates the absence of passive demethylation process. Therefore the presence of the active mechanism responsible for alteration of MUC2 promoter methylation appears to be better fitting in the described model.

5.4 The role of methylation in the development of the mucinous carcinoma

The level of methylation in mucinous tumours similar to that in normal goblet cells suggests that mucinous tumour cells may derive from stem cells undergoing goblet cell-like differentiation. Alternatively, the low methylation of MUC2 gene promoter in mucinous carcinomas could be a true carcinoma-associated change as observed for MAGE-1 gene promoter methylation in melanomas [122]. *MUC2* gene suppression and a simultaneous overexpression can, however, be observed in the same colon cancer. Indeed, DNA from a microdissected nonmucinous region of a mucinous tumour showed *MUC2* promoter methylation practically identical with the nonmucinous phenotype at all the investigated sites (data not shown), i.e. a low as well as a high methylation of this gene can be associated with tumour growth. It is therefore unlikely that the low or high methylation of *MUC2* promoter represent different carcinogenic lesions. It can be suggested that the extent of *MUC2* gene promoter methylation reflects distinct differentiation pathways on which carcinogenic lesions are superimposed.

The concept that mucinous and nonmucinous colorectal tumours develop along two independent differentiation pathways might also explain the hitherto puzzling observation of a different frequency of late genetic lesions, e.g. p53 mutations [21] or DCC loss in

these two types of cancer. These genetic differences may be due to a different susceptibility of secretory (goblet) and absorptive (columnar) cells to the effects of mutagens and/or to a different repair capacity of these cell types.