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Differences in C/EBPs in normal tissue and papillomas of the larynx

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Abstract. Transcription factors belonging to the family of CAAT enhancer binding proteins (C/EBPs) programme differentiation in a wide variety of cells. We asked about the expression of C/EBPs in squamous epithelium. Using immunohistochemistry, C/EBPα and C/EBP*β* were easily detected in tissue sections from normal larynx or laryngeal papillomas, benign tumours with a papillomavirus aetiology and characterized by abnormal differentiation. A temporal expression of these C/EBPs occured as keratinocytes differentiated. In both tissues, C/EBP*β* appeared to be exclusively nuclear. In normal tissue, the greatest amount of C/EBP*β* was present in the spinous layer, and much less occurred in the granular layer. The basal layer of the laryngeal papillomas contained the most C/EBP*β*. Less was present in the spinous layer. Little C/EBP*β* was in the granular layer. Much C/EBPα was in the cytoplasm in both tissues. In normal tissue, nuclear C/EBPα staining was virtually absent in the basal layer, and became present in the spinous layer. Nuclear C/EBPα was randomly distributed in all layers in papillomas. Using immunoblots and Southwestern blots, we detected abundant truncated isoforms of C/EBPα in the papillomas. Since differentiation of many tissues is determined by the relative amounts of different C/EBPs, our data supports a role for C/EBPs in the differentiation of squamous epithelium.

Laryngeal papillomas are benign epithelial tumours whose aetiological agent is the human papillomavirus (HPV), usually HPV type 6 or 11 (Gissmann *et al.* 1983). These HPVs cause similar tumours in the oral cavity, reproductive (genital) tract and respiratory tract. Abnormal differentiation of epithelium characterizes these papillomas. The basal layer usually appears normal, but the spinous layer is hyperplastic. Koilcytes are often present (Abramson, Steinberg & Winkler 1987). Additionally, a number of differentiation-specific proteins are changed in these laryngeal papillomas. A reduction of keratin 13 and an absence of filaggrin occur in the papillomas. *ψ*-3, a marker for abnormal differentiation, occurs in these papillomas but not in normal laryngeal epithelium (Steinberg *et al.* 1990).

Expression of HPV DNA depends on differentiation of keratinocytes (Stoler *et al.* 1989, 1990). In turn, the characteristic pathology of these papilloma lesions depends on expression of HPVs (Maran *et al.* 1995). The viral DNA is relatively quiescent in basal cells. Increased

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viral transcription and replication occur in the very differentiated layers (Stolar *et al.* 1989, 1990). The mechanisms whereby little expression of HPV DNA occurs in basal cells but can be activated in differentiated cells are not known. Most infections with HPVs are characterized by presence of HPV DNA with little or no expression of this DNA and no pathology (Steinberg *et al.* 1983, Ferenczy *et al.* 1985, Maran *et al.* 1995). Therefore, we speculate that some aberrant differentiation of keratinocytes predisposes infected keratinocytes to express viral DNA.

We know that the transcription factor C/EBPβ (NF-IL6) is a negative regulator of HPV11 expression in normal cultured keratinocytes (Wang *et al.* 1996). The significance of this observation is that the C/EBP family of transcription factors programme differentiation in a wide variety of tissues including adiopocytes (Cao, Umek & McKnight 1991, Umek, Friedman & McKnight 1991), myelomonocytes (Scott *et al.* 1992, Katz *et al.* 1993) and hepatocytes (Isshiki *et al.* 1991, Michoulon *et al.* 1992). In adipocytes, C/EBPβ is most abundant in pre-adipocytes while C/EBPα is most abundant in mature adipocytes (Cao *et al.* 1991). Over-expression of C/EBPα causes overt expression of adipocyte morphology in adipoblasts (Umek *et al.* 1991). Antisense to C/EBPα RNA prevents maturation to adipocytes (Lin & Lane 1992). Little information exists as to whether C/EBPs control differentiation in keratinocytes.

The theme of C/EBPs is that the relative amounts of different C/EBPs, which form homo and heterodimers with each other, control differentiation. The relative amounts of C/EBPβ to C/EBPα are important in adipocyte differentiation. Isoforms of an individual C/EBP also control activities of C/EBPs. The intronless genes of C/EBPα and C/EBPβ code for several proteins which are generated post-transcriptionally by leaky ribosomal scanning (Ossipow, Descombes & Schibler 1993, An *et al.* 1996). Such isoforms have identical C-termini but are truncated at the N-termini. The use of alternate AUG start sites that generate these isoforms is dependent, at least in part, on response to cytokines (An *et al.* 1996) and steroids (Aught, Liau & Rosen 1995). The importance of these isoforms is that they have significantly different regulatory functions. Full length C/EBPα (a 42 kDa protein) has antimitotic activity (Hendricks-Taylor & Darlington 1995). A truncated version (30 kDa) fails to interfere with adipocyte cell proliferation and has an attenuating effect on the differentiation of specific genes that the p42 activates (Lin *et al.* 1993). Isoforms of C/EBPβ (LAP, a 32 kDa protein and LIP, a 20 kDa protein for murine C/EBPβ) have very different properties. LIP antagonizes activities of LAP (Descombes & Schibler 1991). Significantly, neoplastic transformation of mammary epithelial cells associates with the expression of LIP (Raught *et al.* 1996).

In this study, we examined expression of C/EBPα and C/EBPβ in tissue sections from epithelium of normal larynx and from laryngeal papillomas. We found that a temporal expression of C-EBPβ and nuclear C/EBPα occurred as cells differentiated. We found differences in their expression between normal larynx and papillomas. Additionally, more truncated isoforms of the C/EBPα occurred in papillomas.

MATERIALS AND METHODS

Reagents and materials

Polyclonal antibodies made with synthetic peptides to the carboxyl termini of C/EBPβ (amino acids 258–276 of rat C/EBPβ) and C/EBPα (amino acids 253–265 of rat C/EBPα) and the peptide used to make antibodies againt C/EBPβ were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Human papilloma tissue, normal laryngeal tissue and newborn foreskin tissue were surgery discards performed for other reasons at Long Island Jewish

Medical Center. Tissues for immunohistochemistry were frozen immediately in OCT compound from Miles Scientific. Tissues for protein analysis were flash frozen in liquid nitrogen after removing any visible connective tissue.

Immunohistochemistry

The immunohistochemistry procedure was that used by Boudreau, Blais & Asselin (1996) for examination of C/EBPs in other tissues. Briefly, frozen tissue samples were cut into 8-μm thick sections, air dried and fixed in cold acetone using standard procedures. Sections were incubated with normal rabbit IgG (0.20 μg/ml) as a negative control, anti-C/EBPα (0.20 μg/ml) or anti-C/EBPβ (0.20 μg/ml) overnight at 4°C and processed for immunoperoxidase using Vectastain-ABC reagents from Vector Laboratories (Burlingame, CA). An additional negative control was anti-C/EBPβ pre-incubated with the synthetic peptides described above.

Quantitative evaluation for nuclear C/EBPβ

After immunohistochemistry detection for C/EBPβ, the relative intensity of staining of individual nuclei was determined using the IS-1000 digital imaging system (Alpha Agnatic Corporation, San Leandro, CA) with associated software. Two normal laryngeal tissues and three laryngeal papillomas were evaluated. At least two sections from individual tissues were evaluated. The immunohistochemistry processing was done at the same time for all tissues.

Protein extracts

Preparation of total protein extracts from frozen tissue or nuclear extracts from cell lines was described by us previously (Auborn *et al*. 1989). After pulverizing frozen tissue in a Braun dismembrator, proteins were extracted in a high salt buffer and precipitated with ammonium sulfate using standard methods. Proteins were extracted from the nuclear pellet of cultured cells (after lysing cells in hypotonic solution) identical to pulverized tissue. Protein concentration was determined using reagents and protocols of the protein assay kit from BioRad Laboratories, Hercules, CA.

Western analysis

Standard procedures were used. Fifty micrograms of protein extract were electrophoresed on a 12% denaturing SDS polyacrylamide gels, transferred in Immobilon-P membranes (Millipore Lab Products, Bedford, MA) and preincubated with 10% newborn calf serum and 0.05% Tween 20 in TBS (25 mM TRIS, pH 8.0, 137 mM NaCl, 5 mM KCl) followed by incubation with anti-C/EBPα or C/EBPβ at 0.2 μg/ml. After extensive washing, membranes with developed with peroxidase-labelled goat anti-rabbit IgG (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) and enhanced with chemiluminescence reagents from Amersham Pharmacia Biotech Inc., Piscataway, NJ.

Southwestern analysis

Assays were as previously described by us (Auborn & Steinberg 1991). Protein extracts (50 μg of nuclear extract or 100 μg of tissue extract) were electrophoresed in 12% SDS polyacrylamide gels, transferred to nitrocellulose and denatured in 6 M guanidine HCl. After stepwise renaturing in binding buffer (250 mM HEPES, pH 7.9, 30 mM $MgCl_2$, 400 mM KCl, 1 mM dithiothreitol) and blocking with 5% non-fat dry milk, DNA binding to the immobilized proteins was in 0.25% dry milk in binding buffer with ^{32}P-labelled double-stranded oligomers

(GCAATAAACAAT) made into concatemers of approximately 200 base pairs (bp). Binding was 16 h at 4°C.

RESULTS

C/EBPα and C/EBPβ are expressed in epithelium of normal larynx and laryngeal papillomas

We examined squamous epithelium from laryngeal tissue. The goal was to determine whether members of this family of transcription factors known as C/EBPs were present. Additionally, we wanted to know how they were expressed within the epithelium. Tissues (surgical discards) were all from different individuals. Normal larynx tissue was from laryngectomies. Benign tumours of the larynx were the papillomatous lesions caused by HPV. Using immunohistochemistry to evaluate tissue sections, C/EBPβ occurred in epithelium from both tissues and was clearly nuclear (Figure 1). A quantitative analysis determined the relative intensity of staining per area in nuclei of basal, spinous and granular layers (Figure 2). Only cells that touched the basement membrane were called basal cells. In normal laryngeal tissue, nuclei of the spinous layer contained the greatest amount of C/EBPβ. The greatest staining occurred in cells adjacent to the basal layer. Nuclei of the granular layer contained the least amount of C/EBPβ. In laryngeal papillomas, the C/EBPβ was decreased in spinous layer, and was further decreased in the granular layer.

C/EBPα occurred in the nucleus and cytoplasm of both tissues (Figure 3). In normal tissue, nuclear C/EBPα appeared to be absent in the basal layer but became dramatically apparent in nuclei in the spinous layer. In previous studies, C/EBPα was detectable in the cytoplasm but not nuclei of cultured foreskin keratinocytes (Wang *et al*. 1996). Nuclear C/EBPα was more randomly distributed within all epithelial layers in the papillomas. Additionally, more C/EBPα appeared to be in papillomas than in normal tissue. This apparent increase in C/EBPα was supported by Western analysis (Figure 4). Clearly, both C/EBPβ and C/EBPα occurred in the epithelium of the larynx, with temporal expression as cells differentiated. Most dramatic was the decline of expression of C/EBPβ in laryngeal papillomas and the abrupt appearance of nuclear C/EBPα in the spinous layer of normal laryngeal epithelium.

Differences between the C/EBP isoforms in laryngeal papillomas

Using immunoblotting (Figure 4), we compared C/EBPα and C/EBPβ in protein extracts made from the tissue of two different laryngeal papillomas, a papillomatous lesion from the trachea, a normal larynx, a normal foreskin and cultured HeLa cells. The tracheal papillomatous lesion resulted from spread of HPV from the larynx. Foreskin tissue and HeLa cells are other sources of keratinocytes that support HPVs. Immunoblotting would help define isoforms of C/EBPα and C/EBPβ since the isoforms have different molecular weights. The results were that no small isoforms of C/EBPβ (expected size of about 20 kDa) were detected in any of the protein extracts except that of HeLa cells. In extracts made from laryngeal papillomas, the size of C/EBPβ was consistent with the larger isoforms (about 30 and 35 kDa). In extracts from normal tissue, most of the C/EBPβ migrated as higher molecular weight proteins. This C/EBPβ was likely a complex with itself or other leucine zipper proteins. C/EBPs form covalent bonds after dimerizing through leucine zippers. Such complexes are not easily separated (Williams, Cantwell & Johnson 1991). C/EBPβ and its association with proteins was different in normal tissues versus the laryngeal papillomas. Multiple isoforms of the human C/EBPα can be generated. The largest would be about 42 kDa. Hence, smaller proteins (less than 42 kDa) are likely to be C/EBPα isoforms.

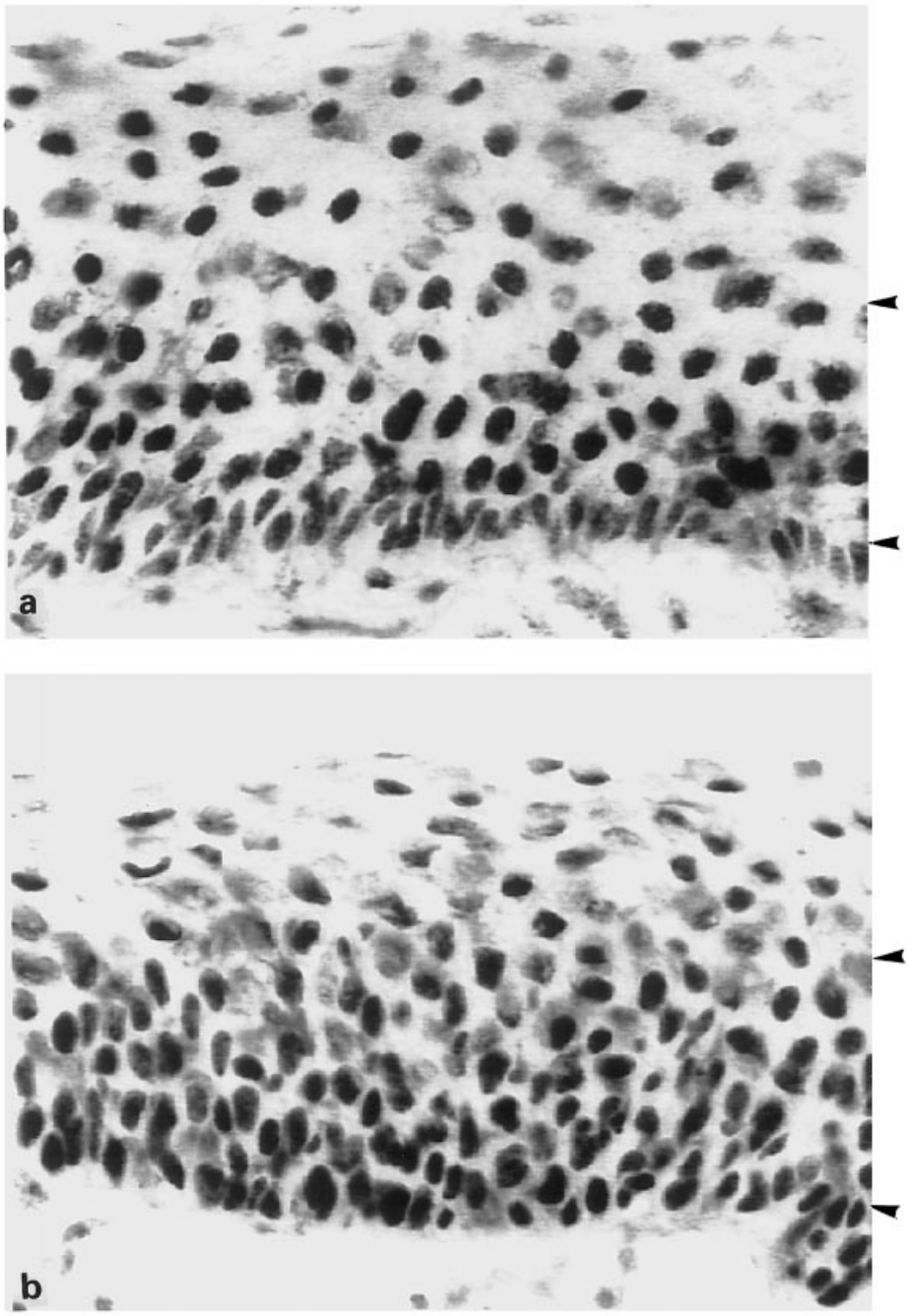

Figure 1. Expression of C/EBPβ in squamous epithelium of the larynx. Immunohistochemistry detection using specific C/EBPβ antibodies were used on cryo-sections from normal larynx (a) and laryngeal papilloma (b). Normal rabbit serum was used in place of C/EBPβ antibodies on a cryo-section of normal larynx (c) and C/EBPβ antibodies preadsorbed with p-19 peptide was used on a cryo-section of foreskin tissue (d). Original magnifications were 12× (a, b and c) and 65× (d). Arrows mark differentiation between basal, spinous and granular layers (a and b and start of basal layer (c and d).

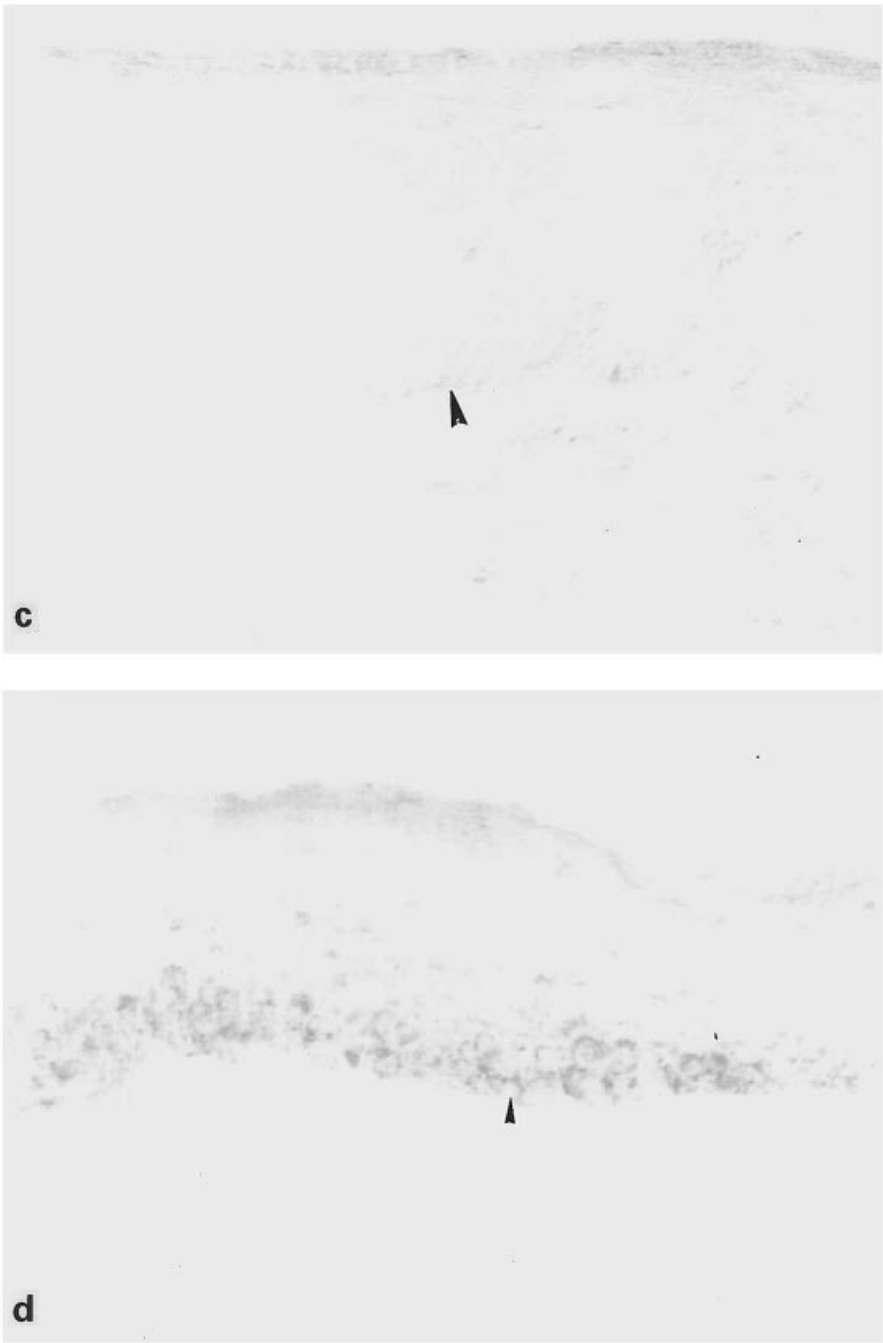

Figure 1. Continued

Striking was the amount of isoforms of aboout 30 kDa in the extracts from laryngeal papillomas. Small isoforms of C/EBPβ and C/EBPα still bind DNA but lack much or all of the 'active' domains of the proteins. Hence, presence of isoforms dramatically impact gene activation or repression. Therefore, we wanted to confirm the presence of these small isoforms in a very different assay determined by their binding to a C/EBP DNA binding motif. Southwestern blots (Figure 5) were done on HeLa nuclear protein extracts and total cell extracts from laryngeal papillomas. A single small protein from the HeLa extract bound this motif. It was probably the small C/EBPβ isoform that was detected in immunoblots. At least three proteins from laryngeal papillomas bound the C/EBP motif. These are consistent with the 14 kDa and 20 kDa isoforms of C/EBPα. The identity of the larger protein may be the 30 kDa isoform of C/EBPα and/or C/EBPβ. A 100 kDa protein binds any DNA sequence and is not specific for the C/EBP binding motif.

DISCUSSION

All the squamous epithelium from the larynx contained easily detectable amounts of C/EBPβ and C/EBPα. The presence and abundance of these transcription factors suggest a function for these proteins in this epithelium. This function is likely to be a role in programming

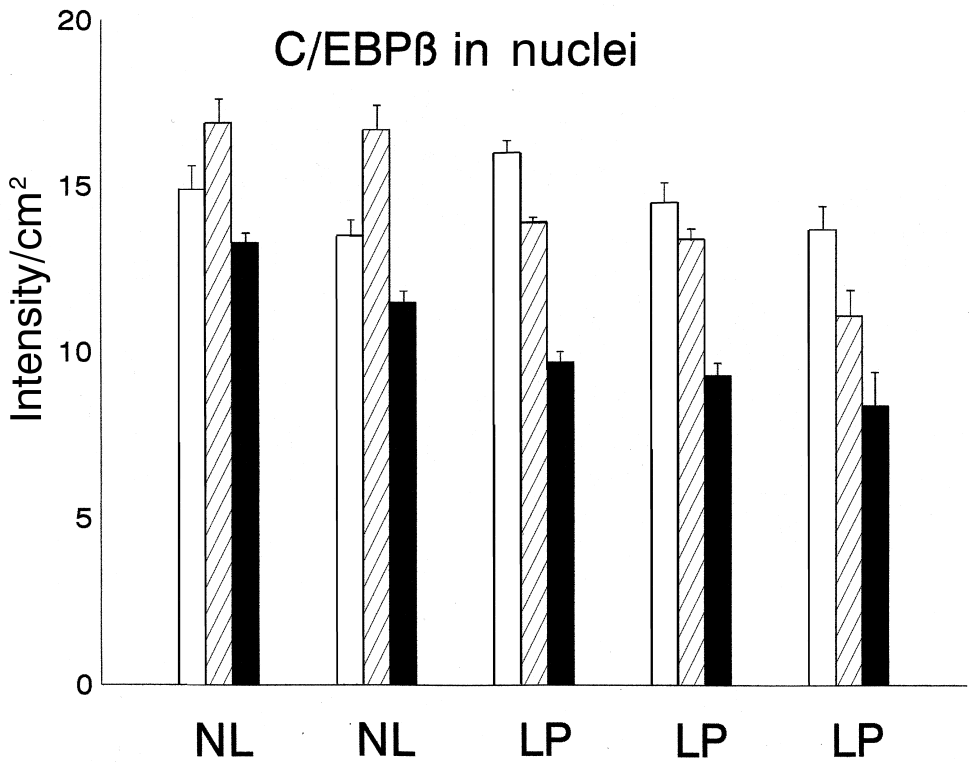

Figure 2. Expression of C/EBPβ in basal, spinous and granular layers in epithelium of the larynx. The relative intensity of nuclear C/EBPβ was determined in the basal, spinous and granular layers in tissue from two normal laryngeal epithelium (NL) and three laryngeal papillomas (LP). □, basal; ▨, spinous; and ■, granular.

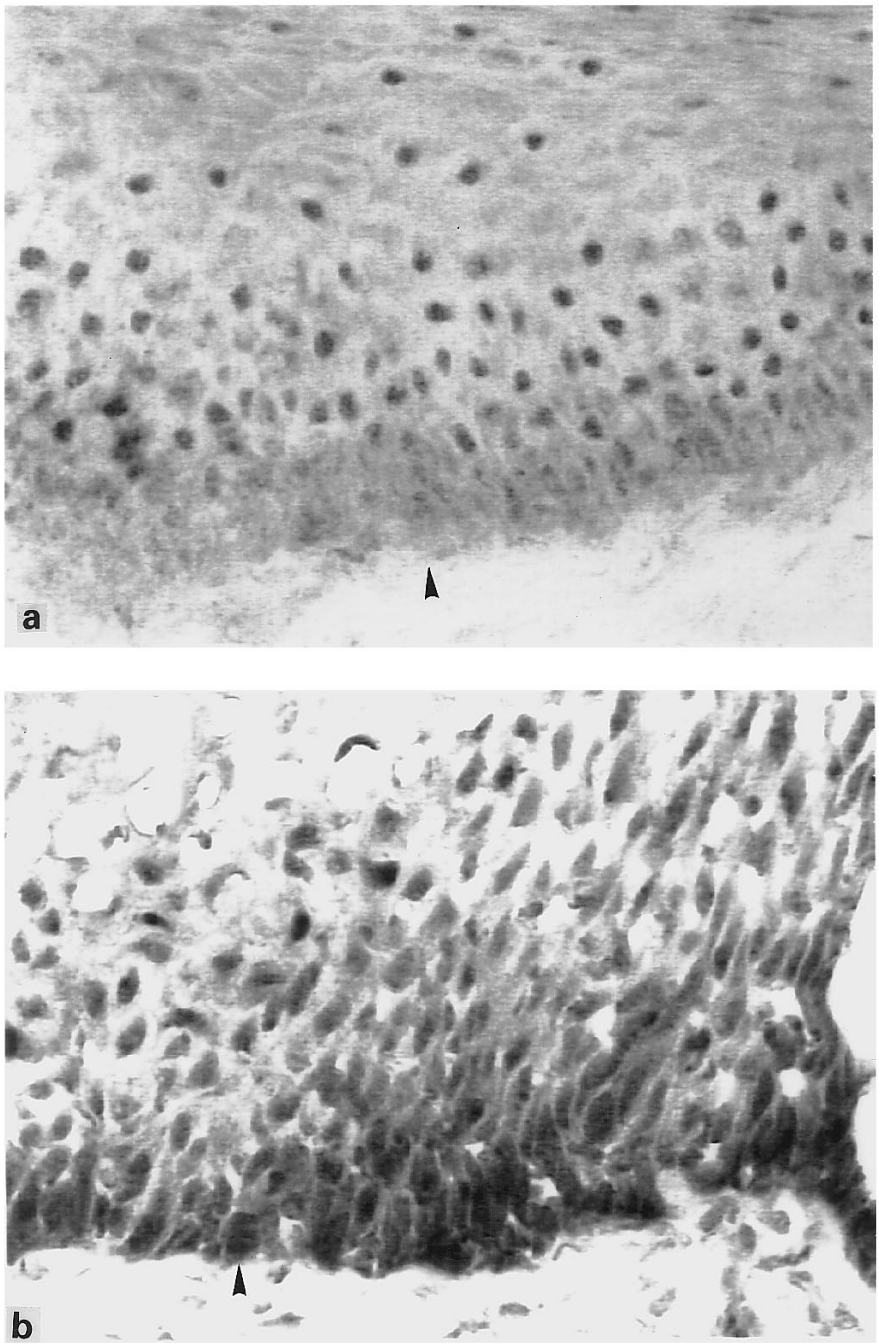

Figure 3. Expression of C/EBPα in squamous epithelium of the larynx. Immunohistochemistry detection using specific C/EBPα antibodies were used on cryo-sections from normal larynx (a) and laryngeal papillomas (b). Original magnifications were 128×. Arrows mark start of basal layer.

differentiation. The relative amounts of nuclear C/EBPβ and C/EBPα changed as cells differentiated. Particularly striking was a rise and fall of C/EBPβ followed by an abrupt increase in nuclear C/EBPα in normal epithelium. Moreover, the distribution of these C/EBPs differed between epithelium of normal larynx and laryngeal papillomas. Both C/EBPα and smaller isoforms of C/EBPα were increased in the laryngeal papillomas. Since laryngeal papillomas have an abnormal differentiation and have differences in C/EBPs, these observations support our hypothesis that C/EBPs influence differentiation of squamous epithelium.

Based on our observations, we can speculate how C/EBPs would help programme differentiation of keratinocytes. In the normal differentiation of this epithelium, we can make parallels to the differentiation of adipocytes. C/EBPβ predominates in cells in the most undifferentiated cells but is dramatically decreased in the more differentiated cells. Cytoplasmic C/EBPα moves to the nucleus as cells start to differentiate thus changing the ratio of C/EBPβ to C/EBPα. Expression of specific genes are determined by the ratio of these C/EBPs.

Enhanced expression of papillomavirus, the aetiological agent of laryngeal papillomas depends on differentiation of keratinocytes. However, virus expression must also depend on abnormal differentiation. In the normal differentiation of squamous epithelium, cells stop replicating. However, papillomavirus requires the cellular replication machinery for its own replication. We can also speculate how C/EBPs could facilitate enhanced transcription and replication of papillomaviruses. C/EBPα (full-length) is antimitotic and activates transcription of differentiation specific genes. Significantly, abundant truncated C/EBPα exists in these

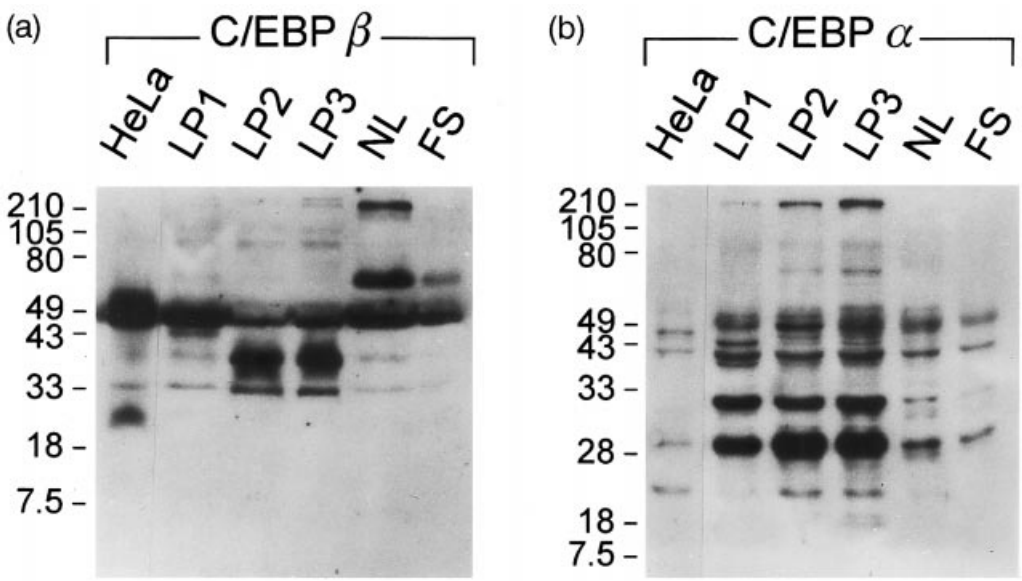

Figure 4. Western blot analysis for C/EBPβ and C/EBPα from squamous epithelium of the upper respiratory and genital tract. Protein nuclear extracts (50 μg) from HeLa cells or from tissue were electrophoresed in denaturing gels and developed with immunoblotting with using antibodies against C/EBPβ (a) or C/EBPα (b). LP1 was from a tracheal papilloma, LP2 and LP3 were from laryngeal papillomas, and FS was from a newborn foreskin tissue. All tissues were from different individuals. Molecular weight (kDa) of markers is indicated.

papillomas. These truncated C/EBPα isoforms lack antimitotic activity (Hendricks-Taylor & Darlington 1995). In particular, the abundant 30 kDa isoform would not only lack the antimitotic activity but still maintain some differentiation activities of C/EBPα (Lin *et al*. 1993). C/EBPα isoforms, that do not have antimitotic activity, would allow for viral replication. Separately, C/EBPβ inhibits expression and replication of HPV (Wang *et al*. 1996). In laryngeal papillomas, C/EBPβ decreases more rapidly as cells move away from the basal layer. More C/EBPα is present as determined by both immunohistochemistry and Western analysis, and C/EBPα would sequester C/EBPβ, thereby increasing viral expression. Different from normal epithelium, nuclear C/EBPα is detectable in cells within the basal layer. Hence, the C/EBP profile detected in laryngeal papillomas would be consistent of an abnormal differentiation that would be favourable for papillomavirus expression and replication.

While differences might be expected between tissues from different individuals, it is interesting that laryngeal papillomas (LP2 and LP3) used in the immunoblots were from papillomas on the vocal chords from different patients and derived from squamous epithelium. These tissues had virtually identical profiles of C/EBPs. The profile of C/EBPs did

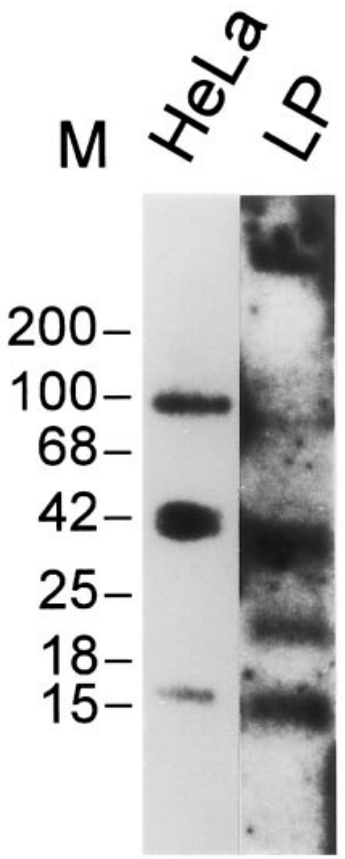

Figure 5. Southwestern blot analysis of proteins interacting with C/EBP DNA binding motif. Protein nuclear extracts from HeLa cells (50 μg) or protein extract (100 μg) from laryngeal papillomas (LP) were electrophoresed, renatured and bound to concatemers of a C/EBP binding motif as described in methods. Molecular weight (kDa) of markers is indicated.

differ in LP1 versus the other papillomas, and LP1 was a tracheal papilloma and hence, derived from respiratory epithelium. This observation raises the possibility that the profile of C/EBPs may be different in different types of epithelium.

The detection of the truncated C/EBPβ in HeLa cells may mimic breast epithelium where neoplastic conversion correlates with the detection of this truncated isoform (Raught *et al.* 1996).

Extended studies need to be done to define the role of C/EBPs in the differentiation (normal and abnormal) of squamous epithelium. The importance of these early studies is that C/EBPs are modulated by cytokines, hormones and other influences. Therefore, it should be possible to modulate C/EBPs favourably to treat or prevent abnormal proliferation of the squamous epithelium.

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