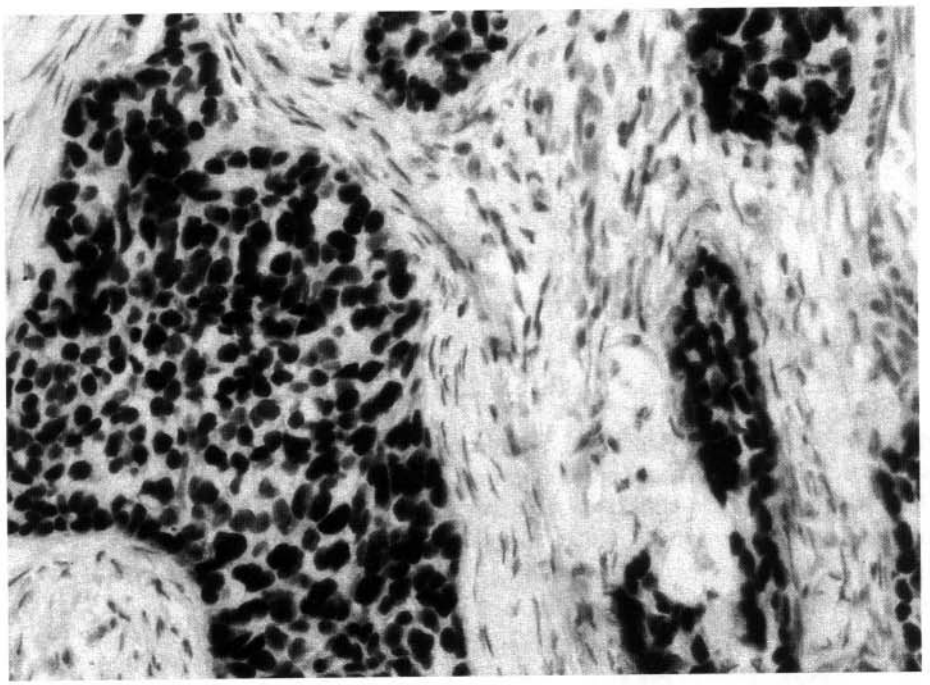

Fig. 3. Detection of p53 posttranslationally modified by phosphorylation at Ser^{392} in ovarian cancer with a phospho-specific antibody S-P-3.1. For antigen retrieval, formaldehyde-fixed tissue was incubated at 97°C in citrate buffer for a period of 30 min. Magnification 200x.

Specificity

First, to test the specificity of MAbs for posttranslationally modified protein at Ser^{392}, p53 over-expressed in baculoviruses and bacteria was analyzed. Western blots using purified p53 protein were performed using S-P-1.1, S-P-2.1 and S-P-3.1 MAbs in denaturing conditions. All three antibodies very strongly recognize the p53 protein over-expressed in baculoviruses; antibodies S-P-1.1 and S-P-2.1 also recognize, with different affinity, the protein over-expressed in bacteria (data not shown). To test the specificity of MAbs on protein posttranslationally modified only on the Ser^{392}, bacterial p53 protein was *in vitro* phosphorylated at this site with CKII. Western blot analysis employing S-P-1.1, S-P-2.1 and S-P-3.1 MAbs detected *in vitro* phosphorylated p53. Antibodies S-P-1.1 and S-P-2.1 did not fail completely to detect non-phosphorylated bacterially expressed p53 (Fig. 2A). To demonstrate the same levels of p53 in each reaction, the non-phosphorylated and phosphorylated p53 were immunoblotted with monoclonal antibodies specific to the C-terminal domain DO-1 (Vojtesek et al., 1992), and polyclonal sera CM-1 (Midgley et al., 1992) recognizing many epitopes on the p53 protein. All three newly developed MAbs recognize well the phosphorylated form of the peptide; two of them (S-P-1.1, S-P-2.1) can also bind, with lower affinity, to non-phosphorylated protein p53 produced in bacteria; the antibody S-P-3.1 binds specifically only to p53 modified at Ser^{392} (Fig. 1). The specificity of the S-P-3.1 antibody to phosphorylated p53 was additionally proved using the experiments with phosphatase treatment (Fig. 2B) in order to remove phosphate groups from different sites of the p53 molecule. After this treatment the antibodies S-P-1.1 and S-P-2.1 still recognized a small proportion of non-phosphorylated p53 protein, whilst S-P-3.1 was not capable of reacting with dephosphorylated p53 at all.

The process of constructing a MAb against a specifically phosporylated epitope does not exclude the possibility of obtaining an antibody reacting with different affinity with both the phospho- and non-phospho-form of an identical protein. Therefore, careful screening of specific hybridomas is of prime importance for eliminating even weak cross-reactivities and for obtaining a pure and site-specific antibody.

Properties

Antibodies S-P-1.1 (IgG1), S-P-2.1 (IgG1) and S-P-3.1 (IgG1) can recognize phosphorylated p53 at Ser^{392} under denaturing and non-denaturing conditions with a different degree of cross-reactivity, the S-P-3.1 being the most specific. All three antibodies can be used for analysis of the cellular p53 status by ELISA, immunoblotting and immunohistochemistry (Fig. 3). The antibody S-P-3.1 seems to be biologically most relevant for experimentation, since it does not display any cross-reactivity with the non-phosphorylated form of p53.

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