Review

Cancer: Is There Involved a Bunch of Culprits, One Culprit, or Something in Between?

(oncogenesis / single-hit / multi-hit / v-*src* / c-*src* / oncogene activation / oncogene down-regulation)

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In the last decade, a good evidence has been collected demonstrating that progression of many tumours is accompanied by serial changes involving oncogenes, antioncogenes and additional genes responsible for DNA repair, genes influencing cell survival, cell signalling and cycling, as well as genes contributing to the cell release from the control exerted by its neighbours, ensuring angiogenesis and putative, not yet defined gene activities, as thoughtfully reviewed by Klein (1996, 1998). There is no doubt that such a complex view reflects many cases of tumour progression. However, there remains an important issue of the hierarchy of these events, especially of what change(s) are crucial for tumour formation and its maintenance, because they should represent primary therapeutic targets.

In this short commentary I would like to discuss the complexity of an oncogene function in relation to tumour progression and maintenance, using as an example the first defined oncogene v-*src*. This oncogene has been molecularly defined in chicken Rous sarcoma virus (RSV) strains (Stéhelin et al., 1976 a,b), where it constitutes a part of the viral genome. It is known that v-*src* represents a truncated version of the normal cellular gene c-*src*, which codes for protein tyrosine kinase involved at various levels of intracytoplasmic signal transduction.

A plethora of data have been collected showing the ability of v-*src* to transform cells *in vitro*. But even more striking are the results in which v-*src*, when inoculated as cloned DNA, triggers oncogenesis *in vivo*. A series of these studies were started by Fung et al. (1983), who employed subcloned DNA fragments accommodating v-*src* equipped at its 3' end only with either full long terminal repeats (LTRs) or with the U3 LTR part carrying only the enhancer sequences. Despite the fact that these constructs were lacking the 5'end regular promoter-enhancer (because 5'end LTR was stripped off), they successfully induced tumours in the chicken line $15I_5$ x 7_1. However, the fast-appearing tumours regressed. An additional problem resulted from the presence of Rous-associated virus-O encoded by *ev* 2 locus (RAV-O) in this chicken line which, as indirectly indicated by the authors, contributed to the spread of v-*src* mRNA and was probably responsible for the oligoclonality of some tumours. However, the authors also noted that tumours were produced in chickens lacking RAV-O and other avian leucosis virus (ALV) genomes integrated in avian germ-line cells (*ev* loci).

More unambiguous results were obtained by Stoker and Sieweke (1989), working with the Spafas chicken line and a plasmid composed of neomycin resistance (*neo*) and v-*src* genes flanked at both ends with LTRs. This vector was then introduced in a chicken helper cell line equipped with retroviral genes coding for products required for virion formation. In such a helper line the vector RNA transcript became encapsidated in virions. After infection of chickens, aggressively growing clonal tumours were obtained, which gave rise, in one quarter of animals, to metastasis formation. Because the LTR, *neo*, v-*src*, LTR vector was packaged via helper cells into virions, it was possible to estimate a relationship between the vector efficiency to be expressed *in vitro* (foci-expressing v-*src* were detected by anti-v-Src antibody) and its tumorigenicity *in vivo*. It was found that 30 to 3000 such foci-expressing units led to formation of tumours in all injected animals. This documents a high sensitivity of *in vivo* oncogenicity testing. The use of a retroviral vector produced the complication that in 20% of tumours, a replication-competent virus was found that arose by recombination of retroviral vector sequences with retroviral replicative genes present in a chicken helper cell line, but it did not seem to put in question the overall results. An additional observation was made showing that the v-*src* from the original vector was often spliced out and induced tumours by itself.

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Abbreviations: LTR(s) – long terminal repeat(s), MHC – major histocompatibility complex, RAV-O – Rous-associated virus-O encoded by *ev* 2 locus, RSV – Rous sarcoma virus, UTR – untranslated region.

The controversy about v-*src*-induced tumour regression versus progression has been resolved by Mike Halpern's group and our group. Using RSV subgenomic cloned DNA comprising v-*src* equipped at the 3' end with an enhancer or LTR (Halpern et al., 1991), it has been demonstrated that tumour progression or regression was dependent upon the chicken line inoculated with v-*src* DNA (the SC line allows tumour progression in contrast to the T line) (Taylor et al., 1992).

Furthermore, using congenic chickens differing only in subregions of the B locus, the avian equivalent of the major histocompatibility complex (MHC) in mammals, again particular B alleles ensured a different tumour fate (Plachý et al., 1994). In our experiment we employed the molecularly cloned LTR, v-*src*, LTR provirus derived from the H-19 hamster tumour line (Bodor and Svoboda, 1989), the sequence of which matches exactly the reverse transcript of v-*src* mRNA. Proviral DNA was inoculated to congenic CC and CB chickens differing only in the B allele (B^4/B^4 and B^{12}/B^{12}, respectively). The CC line turned out to ensure tumour progression in contrast to the CB line, where tumours tended to regress (Svoboda et al., 1992). In addition, while back-crossing F1 hybrids of both chicken lines, the progressor or regressor phenotype segregated with the corresponding B allele (Plachý et al., 1994), which provided a formal proof for association of the studied phenotypes with a particular B allele.

Because MHC decides about the strength of the immune response to antigenic epitopes, the CB chickens in which tumours regressed were rechallenged with increased amounts of LTR, v-*src*, LTR proviral DNA or with an oncogenic dose of RSV. In both cases we observed a striking resistance to rechallenge, similarly as Wisner et al. (1991) have found in their system. These data clearly indicated that immunity against the v-Src oncogene product has been involved. That this is the case was demonstrated in further experiments, in which adoptive transfer of immunity (Plachý et al., 1994) and $CD8^+$ T lymphocyte-mediated cytotoxicity directed specifically against v-Src was revealed. The key role of $CD8^+$ T lymphocytes as effector cells responsible for anti-v-Src immunity was established using anti-CD8 monoclonal antibodies (Svoboda et al., 1996). In addition, the specificity of anti-v-Src rejection immunity was verified by showing that the immunity was specifically directed against v-*src* tumours, but not against v-*src* PR2257-induced tumours, the latter being also highly oncogenic but not matching any of v-*src* mutations, and therefore sharing no antigenic epitope with v-*src* (Geryk et al., 1989). Interpreting the significance of anti-v-Src immunity for v-*src* oncogenicity, it can be concluded that this immunity is established only on a certain histocompatibility background and does not prevent the outgrowth, but tumour progression.

What of these results puts in evidence a possible v-*src* single-hit oncogenic effect? Most straightforward data were obtained with several subclones accommodating only the v-*src* coding region, which were found to be highly tumorigenic on a certain MHC background. Generally, the amount of DNA injected in an animal was in the range of 1 μg. Such an amount should accommodate about 10^{11} molecules of a size corresponding to v-*src* with LTRs. According to our unpublished data, 0.05 μg of v-*src* DNA produce tumours in almost all animals, which points to at most 10 molecules. The efficiency of DNA integration and expression *in vivo* is not known, but it should be relatively low. Nevertheless, it is needed to measure the lowest amount of DNA still active for tumour formation and, in parallel, to quantitate the v-*src* expression in cultured fibroblasts transfected in the same way. Actually, Stoker and Sieweke (1989) provided such a comparison for retroviral vector containing v-*src*, and according to their best estimates, 30 v-*src* expression units produced a progressing tumour. This finding speaks best in favour of the v-*src* ability to trigger oncogenesis in the absence of other additional changes.

If this is so, then v-*src* should be endowed with several abilities required to fulfil the criteria for a single step-acting oncogene:

1. v-*src* is an efficient cell-transforming agent responsible for morphological transformation resulting in a loss of oriented actin filaments, decrease of cell adhesion, and in the ability of anchorage-independent growth (reviewed Svoboda, 1986).
2. v-*src*, due to its constitutively increased protein kinase activity, reinforces the main signalling pathway from receptors of growth factors (Abram and Courtneidge, 2000; Gonfloni et al., 2000). In addition, it can activate, through unknown steps, the c-*myc* protooncogene (Barone and Courtneidge, 1995) (see Fig. 1).
3. v-*src* acts as a mitogen enabling transition steps from the G0 to the S phase and from G2 to mitosis (Roche et al., 1995; Wyke et al., 1995; reviewed by Taylor and Shalloway, 1996) (see Fig. 1).
4. v-*src* developed two known strategies to ensure the escape of transformed cells from apoptosis, which was shown on different models to act as an important control over transformed cells. One strategy is based on activation of a *bcl*-2-related gene producing a potent antiapoptotic factor (Gillet et al., 1995; Mangeney et al., 1996), and the second one takes advantage of an efficient phosphoinositide-3-OH kinase (PI3-kinase) binding to Src via its SH-2 domain, which triggers the PI3-kinase and, consequently, protooncogene Akt (PKB) activation by phosphorylation. Activated Akt is presumed to act in an antiapoptotic way (reviewed Hemmings, 1997; Toker and Cantley, 1997). Recently, more direct evidence suggesting the pathway in which PI3-kinase is activated by v-Src in association with the adapter protein Cbl and functional protein-tyrosine phosphatase Shp-2 was obtained (Hakak et. al., 2000). Such an unaltered pathway activates Akt which, as shown, protects v-*src*-transformed cells from experimentally produced apoptosis (see Fig. 1).