Review Article

Cell Association in Rous Sarcoma Virus (RSV) Rescue and Cell Infection

(retrovirus / RSV / transformation / transfection)

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Abstract. In my article I tried to present the results of early experiments suggesting a significant role for cell association in Rous sarcoma virus transformation of non-permissive cells and revealing that infectious virus can be efficiently rescued from such cells by their fusion with permissive chicken fibroblasts.

Introduction

In the course of recent years, the mode of retrovirus transmission has been subjected to repeated re-evaluation. It has become obvious that close cell-to-cell contact represents a very efficient means for virus transmission (reviewed in Sattentau, 2010; Wurdinger et al, 2012; Costiniuk and Jenabian, 2014) and is employed by virus families such as Herpesviridae, Paramyxoviridae and Retroviridae. In the latter case, a prominent role is played by HIV, where virus transmission by cell association was found to be increased by at least three orders of magnitude over free virus infection (Zhong, 2013).

Avian alpharetrovirus transmission is governed by well-defined cellular receptors, which bind the retroviral surface (SU) virus envelope domain, thus allowing virus penetration into the cell. Depending on the viral receptor nature, alpharetroviruses were divided into several subgroups (Weiss, 1993).

Successful transmission of a prototype alpharetrovirus – Rous sarcoma virus (RSV) – to rodents (reviewed in Svoboda, 1986) created a new experimental situation, indicating the possibility that RSV, under the conditions of close cell-to-cell contact, can bypass the receptor requirement and transform mammalian cells. Furthermore, the RSV genome integrated in non-permissive rodent cells is in most cases not fully expressed, but virus production can be activated by cell association with permissive chicken fibroblasts triggering cell fusion between rodent and chicken cells.

Cell association employed for early RSV transmission studies

Transmission of RSV has a long history, which can be traced to the origin of the virus. The original tumour that Peyton Rous had found in Plymouth Rock chicken and called sarcoma No. 1, was not transmissible by cell-free extract but only by transfer of tissue fragments to chickens (Rous, 1910). The virus was then successfully isolated from the first in vivo passage of sarcoma tissue (Rous, 1911). Nowadays, it is hard to interpret these findings but there is still the possibility that in addition to other factors, the cell-to-cell contact played a role in the first stages of virus transmission (Svoboda, 2013).

Similarly, a great pioneer of retrovirus hetero-transmissions, Duran-Reynals (1947), successfully transmitted RSV to pigeons by means of RSV-infected chicken tissues.

By analogy, Svet-Moldavsky (1958) succeeded in tumour induction in rats by inoculating them after birth with minced chicken RSV tissue. However, the aetiology of these tumours remained unclear due to the fact that no signs of RSV genome expression or virus production were detected. Therefore, the RSV involvement in tumour genesis was not proved and this result could be explained by a “hit and run” mechanism.

Such interpretation was abandoned owing to the rat tumour line called XC (Svoboda, 1960, 1962), in which RSV was first detected by XC tissue transfer into chickens where it induced fast growing sarcomas accompanied by metastasis formation. In contrast to XC cells, chicken tumours were transmissible by cell-free extracts and the infectivity was neutralized by anti-RSV antisera. Importantly, XC tumours kept the ability to produce chicken sarcomas until 25th passage in vivo, when this experiment was terminated. At that time, I was motivated to employ tumour tissue transfers for RSV detection by my previous experience that it represented a
most efficient way to passage RSV tumours, before tissue culturing made possible high yield virus production. I should note that the designation XC was derived from the Latin number of the cage where the tumour appeared.

Quest for virus nature in XC cells

Since transforming RSV was obtained only from the XC tumour cell line (one of three tumours tested), it looked as if we were dealing with an artificial situation in which several factors might have interacted to result in tumour production. In order to clarify this possible complex issue, I decided to perform control experiments in which chemical carcinogen 3,4-benzpyrene was employed (Shrigley et al., 1947). The rationale of such experiments was to settle the problem whether RSV might preferentially target a tumour tissue produced by another aetiological agent. Rats injected soon after birth were challenged both with the carcinogen and with chicken RSV sarcoma tissue. Carcinogen-induced tumours displaying hard consistence appeared within a few months. Minced tumour tissue was then injected into chickens and no tumours were produced. I also found no increase of carcinogen-induced tumour frequency in the RSV presence, which signifies no co-carcinogenic effect of both agents. Furthermore, when RSV-induced chicken sarcomas were injected into non-viral rat tumours, no case of RSV acquisition was observed. I therefore refuted the possibility that RSV simply entered the rat cells made malignant by another agent (Svoboda, 1962).

It did not seem probable that the XC tumour that arose after 7-month latency might contain chicken cells, but we wanted to obtain fully convincing evidence. In this respect, caution was warranted because the XC tumour was induced in outbred Wistar rats and could be passaged in young animals only. From additional experiments we learned that in such animals chicken RSV tumours survived and proliferated not more than for two weeks (Svoboda and Grozdanović, 1961). In order to fully establish the XC nature we employed chromosomal analysis, which provided additional evidence that XC cells harbour only rodent chromosomes in the absence of chicken micro-chromosomes (Landa et al., 1962a, b; Svoboda et al., 1962). Similarly, agar diffusion experiments confirmed that XC cells harbour antigen precipitation bands corresponding to those obtained with rat proteins (Svoboda and Gusev, 1962). Collectively, all these results confirmed that the XC tumour arose by RSV-mediated malignant transformation of rat fibroblasts.

Virus rescue

A surprise was waiting for me in relation to the mode of XC RSV production. I encountered this problem when comparing the virus-inducing capacity of intact XC cell with cell suspensions structurally destroyed by three cycles of fast freezing and thawing (Svoboda, 1962). It turned out that only intact XC cells produced RSV-containing tumours in chickens. Having this experience I suggested to Simković to culture XC cells together with chicken fibroblasts, and he also succeeded in RSV recovery under such circumstances (Simković et al., 1962). In those days the notion that RSV requires cell association for its production was almost unacceptable. Despite general disbelief we decided to follow the track according to which mammalian RSV-transformed cells require complementation with permissive cell factors for virus production. As presented at the International Conference on Avian Tumor Viruses in Durham, I took into account both intercellular bridges and cell fusion as possible culprits responsible for virus rescue (Svoboda, 1964).

In the next experimentally achievable step we performed cell fusion experiments in which XC cells were hybridized by inactivated Sendai virus with chicken fibroblasts and we found that this procedure significantly increases virus rescue by co-cultivation (Svoboda et al, 1968a).

During my stay at the Imperial Cancer Research Fund we quantitated cell fusion experiments and established a linear dependence of virus rescue on XC cell amounts (Svoboda et al., 1968b; Svoboda and Dourmashkin, 1969). We established that cell fusion enhances the efficiency of virus rescue 100-fold as compared with co-cultivation and that there exists a good correlation between heterokaryon formation and the degree of virus rescue. These experiments were corroborated by Machala et al. (1970), who showed that isolated XC cells fused with chicken fibroblasts produce virus envelope glycoprotein, and when transferred on a coverslip onto chicken fibroblast culture, give rise to infectious RSV. Comparing a large set of cell lines we found rare cases from which RSV was hard to rescue and which we denominated as poorly vireogenic cells. However, when such cells were pre-treated with 5-bromodeoxyuridine, their virus rescue activity became clearly enhanced (Donner et al., 1974), indicating involvement of a negatively acting cell factor suppressing virus rescue. Some rodent cell lines originally transformed by non-defective RSV strains (Popovic et al., 1977) require not simply fusion with chicken fibroblasts for virus rescue, but such fusion partner cells should be previously infected by a non-transforming ALV (a helper virus). The helper virus can complement mutation in any virus replicative genes or may alleviate additional blocks preventing virus rescue. There remains the question what type of chicken cells can act as a suitable partner for cell fusion. So far, we have identified only chicken fibroblasts as accommodating this role (Svoboda et al., 1971). All presented data that we obtained strengthened the convincing arguments favouring cell fusion as the decisive step leading to virus rescue (Svoboda and Hlozanek, 1970).

Provirus

Now I would like to return to the early 1960s and comment on further extension of the XC story. Knowing that RSV information in unexpressed state is anchored in the XC cell genome, we looked at this unusual situation from the point of view of genetics. Assuming that RSV is integrated in the XC cell population, every single XC cell clone should retain this information. We
therefore designed a cell cloning experiment in which the Sanford’s capillary method (Sanford et al., 1961) was utilized. With a few Pyrex tubes that I had cleaned according to this technique, in Šimkovič laboratory we succeeded in sucking one or a few cells into each capillary. Outgrown clonal cultures retained the same virus rescue activity as the original XC cell population (Simkovic et al., 1963). These results substantiated the interpretation that the RSV genome became an integral part of the rat genome, being integrated in a stable fashion in every XC cell clone. Furthermore, we rechecked the previous finding of infectious RSV absence in XC cells in an extensive manner using high-speed sediments from huge amounts of XC tissue culture fluid and isolating the subcellular fraction corresponding to RSV from 36 grams of XC tissue. No case of virus infectivity was discovered. Similarly, rats regressing XC tumours did not produce any virus neutralization activity and the virogenic activity was not decreased by XC cell cultivation in the presence of anti-RSV serum, thus excluding the possibility that virus reinfection might be involved (Svoboda et al., 1963).

Having proved the permanent presence of non-infectious RSV genome in XC cells we classified them as virogenic cells harbouring integrated RSV provirus. This was proposed, as Howard Temin acknowledged in his Nobel lecture, independently of his observation (Temin, 1976). Steps leading to provirus postulation were also discussed in Svoboda (2003). Logically, virogenic cell lines provided the best model for biochemical evidence of proviruses integrated in them. This was achieved by repeated transfection of chicken cells with XC DNA (Hill and Hillova, 1971). Similarly, single exposure of chicken fibroblasts to DNA from this and an additional hamster virogenic line (Hlozanek and Svoboda, 1972; Svoboda et al., 1972, 1973) resulted in RSV production. As each cell employed was originally transformed by RSV displaying its own subgroup specificity, we also demonstrated serologically that viruses obtained after transfection kept the subgroup of their origin, which established specificity of the transfection results. Despite the fact that transfection experiments unequivocally proved the provirus DNA nature, the question remained whether nuclear or extra-nuclear DNA was involved. Using enucleated virogenic cells and isolated extra-nuclear DNA we excluded the provirus presence outside the nuclear compartment. All these data agreed with the original postulation of provirus as a cell genome integral part (Donner et al., 1974; Svoboda et al., 1975a).

**Other virogenic mammalian cell lines**

For some time, XC cells remained the only mammalian cells harbouring RSV, which on the one hand decreased the credibility of this finding and on the other hand opened the way for the criticism that we were dealing with some exceptional orphan or even artificial situation. We therefore extended our interest to RSV malignant transformation of rat fibroblasts in vitro. Guided by previous experience, for transformation experiments we utilized co-cultivation of RSV-transformed chicken cells with primary rat fibroblasts. Under such conditions, the rat cell transformation was achieved within a week, in contrast to single RSV exposure that produced no changes (Svoboda and Chyle, 1963). The rat species origin of the transformed cells was successfully confirmed both by karyology and by their virogenic properties (Svoboda et al., 1965). Further rodent species, this time Syrian and Chinese hamster fibroblasts, were transformed in a similar way (Vesely and Svoboda, 1965; Hlozanek et al., 1966) and tumours were also induced in hamsters (Klement and Svoboda, 1963; Svoboda and Klement, 1963) and mice (Bubenik et al, 1967), and their virogenic nature was confirmed.

An interesting twist in XC studies was achieved by Klement et al. (1969), who in the course of study of the mouse leukaemia virus (MLV) possible involvement in RSV rescue found that XC cells were highly sensitive to fusogenic activity of this virus, which resulted in later extension of a widely used quantitative MLV assay also to human retroviruses.

Summing up, XC cell studies extended to other rodent species strongly supported the provirus existence, opened the way to characterization of factors involved in cell non-permissiveness to retrovirus infections and indicated clearly that retrovirus entry to non-permissive cells is facilitated by cell-to-cell association. XC cells became a prototype of RSV-transformed mammalian cells and started to be generally used for comparative retrovirus-cell interaction studies as well as for titration of mammalian retroviruses even of human origin.

**Provirus structure**

The advent of restriction enzymes and blotting techniques opened the way to structural characterization of proviruses integrated in rodent cells. The broadest study in this direction was performed using virus rescued by DNA transfection from XC cells. Single-focus progeny was injected into chickens and the minced tumour tissue was then injected to new-born hamsters. Altogether 24 hamster tumour cell lines were derived from individual tumours. Interestingly, 63 % of them retained the entire provirus structure, but 21 % suffered deletion of the 3’gag end and the entire pol gene. In 8 % cases two proviruses were present, one unaltered and one with the deletion mentioned above. Only in one tumour cell line we detected provirus amplification (Svoboda and Lhotak, 1984; Pichrtova et al., 1987, 1989), however, noticed previously in XC cells (Mitsialis et al., 1983). In both provirus amplification cases, proviruses were not amplified in tandem but located in different genome positions. From the studies of provirus representation in cell DNA fractions we learned that the expressed proviruses favour integration in GC-rich genomic regions (Rynditch et al., 1991), which are most active in both transcription and recombination. We should underline that RSV tumorigenic activity in mammals is a rare event and that in many cases we may expect just provirus integration without sufficient oncogene expression.
Therefore, we have to take into account a significant role of the provirus integration site in deciding upon provirus expression.

A set of RSV-transformed tumour lines with a known provirus structure should be helpful in characterization of such genomic sites modifying provirus expression in non-permissive cells.

**RSV transforming gene**

Is this the end of the story? It does not seem so. During the studies of different cases of RSV-induced mammalian cell transformation it has become obvious that they differ in the degree of expression of certain viral genes.

I was attracted by tumours where we found no viral gene expression and from which no infectious virus was rescued using different complementation and fusion approaches (Svoboda, 1968; Svoboda et al., 1975b). Furthermore, one such mouse tumour – RVP, – remained responsible for rejection of RSV tumours, while the tumour-specific transplantation antigen (TSTA) characteristic of RSV tumours (Svoboda, 1965) had been kept. This antigen was more recently identified to be encoded by the transforming (now v-src) gene, and its formation triggered 11 new amino-acid insertion into this oncogene (Plachy et al., 1994, 2001; Svoboda et al., 1992, 1996). In addition, the morphology of the studied mouse cells agreed with that found in other RSV-transformed rodent cells. In the first classification I called the cells harbouring TSTA but lacking any capacity to yield a rescued virus non-productive and put forward the hypothesis that they may contain only the viral transforming gene (which in those days was not named) (Svoboda, 1968). Working with such non-productive mouse line called RVP, we detected, using liquid RNA hybridization, that it contained about one third of the viral genome that might correspond to a putative transforming gene (Svoboda et al., 1977). This opened a new way to approaching an oncogene existence and genesis.

Similarly, viral RNA was detected in RVP, cell clones, but after passages it tended to fall down (Rynditch et al., 1983); however, unspecified proviral DNA persisted in the RVP, cell clones. As this mouse cell line underwent various rearrangements and formation of micro-chromosomes (Sainerova and Svoboda, 1978, 1981), which might have led to tumour cell heterogeneity, provirus alteration included, we decided to establish a new set of tumour cell lines induced in new-born hamsters by minced chicken PR-RSV-C sarcoma tissue (Svoboda, 1981; Geryk et al., 1984). One of such tumours called H-19 corresponded to what we expected for the non-productive category of tumours – which we renamed cryptoviogenic. The H-19 cell culture was analysed by Southern and northern blotting and it was found that it contains only the oncogene v-src, physically defined and named by Stehelin et al. (1976), and gives rise only to v-src mRNA (Svoboda et al., 1983). Later, synthesis of v-src product pp60 in H-19 was revealed (Grofova et al., 1985). Moreover, we succeeded in rescue of transforming virus by H-19 cell fusion with helper virus RAV-1-infected chicken fibroblasts and we detected particles containing only v-src RNA that transformed chicken fibroblasts by integrating the functional v-src gene into them (Svoboda et al., 1986). In the course of virus rescue, in one case we noted a recombination event between v-src mRNA and RAV-1. This E6 recombinant acquired v-src at the region of 17 nucleotide homology between v-src and RAV-1 gag gene and led to incorporation of the left side gag part in front of the v-src gene (Svoboda et al., 1990).

The full nucleotide sequence was obtained after molecular H-19 provirus cloning, which revealed that the provirus is composed of v-src flanked by LTRs (Bodor and Svoboda, 1989). Therefore, such LTR, v-src, LTR provirus should have arisen by v-src mRNA reverse transcription and regular integration of the reverse transcript accompanied by formation of hexanucleotide repeats of flanking cell DNA typical of ALV integration.

That this simplified provirus is biologically active was proved by previous observation of v-src mRNA synthesis extended to the finding of v-src product (pp60) formation (Grofova et al., 1985). Moreover, DNA isolated from the molecular LTR, v-src, LTR clone induced fast-growing sarcomas accompanied by metastasis formation in chickens, which acquired a simplified provirus corresponding to that of LTR, v-src, LTR (Svoboda et al., 1992), thus presenting a final proof of this provirus biological activity.

**Provirus silencing**

The morphological heterogeneity of cell line H-19 led us to the finding of non-transformed revertant cells segregating from the original H-19 cell population. Revertants that lost transformed morphology and tumorigenic activity appeared with the frequency $10^{-3}$ per cell per generation, much higher than could have been attributed to the random mutations (Hejnar et al., 1994). In contrast to transformed cells, the LTR, v-src, LTR proviruses in revertants were highly CpG methylated. Once demethylated by cloning in methylation-deficient bacteria, they reacquired their malignant properties (Hejnar et al., 1994). Characterization of the genomic site in which LTR, v-src, LTR was integrated revealed that it was inserted adjacent to a negative regulatory region (Machon et al., 1996) which had been severely methylated. However, once the active provirus was inserted, a loss of high methylation density took place (Hejnar et al., 1999, 2003). This suggested a complex interplay between the provirus methylation and the cell epi-genome at the site of provirus insertion. Such interplay was evidenced later in our studies of provirus silencing in context of their integration sites (Plachy et al., 2010; Senigl et al., 2012). Epigenetic mechanisms of provirus silencing have been described more recently as general phenomena governing the latency of HIV-1 (Blazkova et al., 2009) down-regulation of endogenous retroviruses (Matouskova et al., 2006; Trejbalova et al., 2011) and inactivation of retrovirus-based vectors. Again, the studies of RSV silencing inspire the anti-methyla-
tion strategies potentially useful in gene transfer and gene therapy (Senigl et al., 2008).

**Summary and outlook**

Looking back, RSV-transformed rodent cells provided a key for understanding the proviral nature of RSV replication intermediate. This was made possible by defining the virogenic state of RSV-transformed mammalian cells, which means that RSV is integrated in the cell genome but its full expression is hampered.

In addition to original XC rat cell lines we described a series of additional rodent cell lines harbouring one or several amplified proviruses, some of which are prone to transcriptional silence accompanied by provirus methylation. Systematic study of some unusual RVS-transformed rodent cell lines prompted the interpretation that only the RSV genome transforming part is integrated in them. Full molecular analysis of this transforming part (now oncogene v-src) has proved that v-src can act apart from other viral genes and that its transcript (v-src mRNA) can be reverse-transcribed to DNA in vivo, integrated into the cell genome and trigger cell transformation.

Is the model of RSV-transformed rodent cells exhausted? It does not look like that. Recently, Lounkova et al. (unpublished) performed detailed analysis of one virogenic hamster cell line, RSCh, and postulated the existence of at least two blocks responsible for RSV expression block, namely at the level of viral RNA export from the nucleus and in late stages of virus formation. The authors provided evidence that in cell fusion rescue experiments, permissive chicken partner cells provide so far undefined cell protein(s) required for viral particle synthesis. That the situation will be more complex is suggested by the finding that infection of chicken cells used for cell fusion with the helper virus or VSVG plasmid transfection enhances virus rescue. However, both factors per se are not sufficient to trigger any virus production in non-permissive cells. We cannot underestimate the value of cell factor(s) required for virus rescue, especially in regard of their role in deciding upon cell permissiveness to retrovirus infection and retrovirus spreading even to distant species.

It is generally known that retroviruses utilize specific receptors for their entry into cells. In the original cases of RSV rodent cell transformation, such receptors were not available for RSV entry into mammalian cells because these cells lack chicken alpharetrovirus receptors. According to Lounková et al. (unpublished), RSV infection of hamster cells proceeded by some non-canonical way, probably either via a new structural rearrangement of the viral env gene or via direct virus transfer by intercellular bridges or by subcellular particles such as exosomes. This virus transfer mode can be accomplished in other viruses and plays a role even in human retroviruses (Sattentau, 2010; Wurdinguer et al., 2012). In fact, direct cell-to-cell virus transfer has already been suggested as a mechanism of rat fibroblast RSV transformation by co-cultivation with RSV-transformed fibroblasts (Svoboda and Chyle, 1963). An analogical path might be considered for explaining the endogenous retrovirus broad spread, especially in face of the fact that endogenous retroviruses are generally equipped with virus envelopes not adjusted for transfer to distant species.

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