Proteomic Analysis of Secretomes of Oncolytic Herpes Simplex Virus-Infected Squamous Cell Carcinoma Cells

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Abstract: Oncolytic herpes simplex virus type 1 (HSV-1) strain RH2 induced immunogenic cell death (ICD) with the release and surface exposure of damage-associated molecular patterns (DAMPs) in squamous cell carcinoma (SCC) SCCVII cells. The supernatants of RH2-infected SCCVII cells also exhibited antitumor ability by intratumoral administration in SCCVII tumor-bearing mice. The supernatants of RH2-infected cells and mock-infected cells were concentrated to produce Med24 and MedC for proteomic analyses. In Med24, the up- and down-regulated proteins were observed. Proteins including filamin, tubulin, t-complex protein 1 (TCP-1), and heat shock proteins (HSPs), were up-regulated, while extracellular matrix (ECM) proteins were markedly down-regulated. Viral proteins were detected in Med 24. These results indicate that HSV-1 RH2 infection increases the release of danger signal proteins and viral gene products, but decreases the release of ECM components. These changes may alter the tumor microenvironment (TME) and contribute to enhancement of anti-tumor immunity against SCC.

Keywords: oncolytic virotherapy; herpes simplex virus; secretome; proteomics

1. Introduction

Oncolytic virotherapy is a novel therapeutic modality that directly induces the lysis of infected tumor cells, and subsequently enhances host immune responses [1–4]. Recent clinical studies demonstrated that oncolytic viral therapy increases cytokines, activates immune responses, and effectively destroys primary and metastatic lesions, even if the virus does not reach distant lesions. Therefore, oncolytic viruses are also considered to be immunotherapeutic agents [5]. Herpes simplex virus type 1 (HSV-1) is one of the most widely studied viruses for the treatment of patients with solid tumors. Oncolytic HSV-1 lacks the neurovirulence gene γ34.5, which is responsible for encephalitis, as a requisite gene modification for safety [6,7].

In microbial infection, pathogen-associated molecular patterns (PAMPs) that exist in diverse organisms, but not in the host, provide exogenous signal regarding the presence of pathogens in the immune system, thereby promoting immunity [8,9]. In contrast, cells release damage-associated molecular patterns (DAMPs) as endogenous signals that alert the immune system to respond to unexpected cell death, microbial invasion, and stress [3,10]. DAMPs may be proteins, nucleic acids, or metabolic products. Protein DAMPs include intracellular proteins such as high mobility group box 1 (HMG1), heat shock protein (HSP), hyaluronic acid, calreticulin (CRT), and S100 protein [3,5]. When solid tumors are infected with oncolytic viruses, the virus mostly induces immunogenic cell death (ICD), resulting in the cell surface exposure of CRT and HSP and release of ATP, HMGB1, uric acid, other DAMPs, PAMPs, and tumor-associated antigens (TAAs) [3,11–13].
Previously, we reported that an injection of oncolytic HSV-1 strain RH2 into squamous cell carcinoma cell (SCC) SCCVII tumors in inbred mice enhanced systemic anti-tumor immunity [14], and that cell death caused by RH2 was immunogenic cell death (ICD) with the release of DAMPs such as ATP and HMGB1 [13]. We also indicated that intratumoral injection of the supernatants of RH2-infected cells suppressed tumor growth, even if infectivity was lost by ultraviolet irradiation, suggesting their ability to enhance antitumor immunity [13].

Secreted proteins comprise an important group of molecules encoded by about 10% of the human genome, and are able to reflect a broad variety of different conditions of the cell [15]. A proteomic analysis of human macrophages infected with HSV-1 demonstrated the release of proteins functionally related to metabolic processes, transport, stress responses, cell death, proteolysis, the extracellular matrix (ECM), and cell adhesion [16]. However, the characteristics of the secreted proteins, secretomes, of oncolytic HSV-1-infected tumor cells have not yet been studied. In the present study, we performed proteomic analysis of secretomes of RH2-infected and mock-infected SCC cells, and compared the protein profiles.

2. Results

2.1. Classification of Released Proteins of Med24 and MedC

The supernatants of RH2-infected SCCVII cells and mock-infected cells were harvested and concentrated to produce samples named Med24 and MedC. Med24 and MedC were sequentially analyzed, and isobaric tag for relative and absolute quantitation (iTRAQ) quantitation was performed for each protein. A total of 1567 proteins were detected in Med24 and 1344 in MedC, with 223 extracellular proteins being increased by RH2 infection. Based on the results of iTRAQ quantitation, MedC proteins spontaneously secreted from SCCVII cells were classified into three categories, i.e., high, medium, and low abundance groups. The numbers of proteins in the high, medium, and low abundance groups were 190, 262, and 892, respectively.

In order to examine the effects of RH2 infection on protein secretion, iTRAQ quantitation data for each protein was compared between Med24 and MedC. Protein release was found to be up- or down-regulated by RH2 infection. In the high abundance group, 29 proteins were up-regulated by more than 1.4 fold by RH2 infection (Table 1). However, a significant increase was observed in 16 proteins. They included filamin alpha, tubulin beta-4B, clathrin heavy chain, t-complex protein 1 (TCP-1) subunit beta, theta, alpha, gamma and delta, bifunctional purine biosynthesis protein (PURH), filamin-C, bifunctional glutamate/proline–tRNA ligase, plastin-3, fascin, coatomer subunit alpha, beta enolase, and tubulin beta-6 chain.

| Accession No. | Protein Name            | MW (kDa) | iTRAQ Quantitation | Fold Change | p-Value |
|---------------|-------------------------|----------|-------------------|-------------|---------|
| Q9QXS1        | Plectin                 | 534      | 79.7 54           | 1.48        | 0.057   |
| B7EAU9        | Filamin, alpha          | 280      | 73.7 49.7         | 1.48        | 0.004 * |
| F66372        | Tubulin beta-4B         | 50       | 54.7 37           | 1.48        | 0.027 * |
| Q9JF1         | Ras GTPase-activating-like protein IQGAP1 | 189 47.7 33.7 | 1.42 | 0.09  |
| Q9J5F3        | Ubiquitin-like modifier-activating enzyme 1 | 118 40 28 | 1.43 | 0.058 |
| Q5XR6         | Clathrin heavy chain    | 192      | 40 26.3           | 1.51        | 0.023 * |
| P9014         | T-complex protein 1 subunit beta | 57 39 23.7 | 1.65 | 0.012 * |
| P63088        | 60-kDa heat shock protein, mitochondrial | 61 32.4 20.3 | 1.59 | 0.22  |
| F42929        | T-complex protein 1 subunit theta | 60 31.3 20.3 | 1.54 | 0.0013 * |
| Q9CWJ9        | Bifunctional purine biosynthesis protein PURH | 64 27.7 17 | 1.63 | 0.021 * |
| P11981        | T-complex protein 1 subunit alpha | 60 25.7 16.7 | 1.54 | 0.0088 * |
| P95640        | ATP synthase subunit beta, mitochondrial | 56 24 17 | 1.41 | 0.02 |
| Q64737        | Trifunctional purine biosynthetic protein adenosine-5` | 108 22.7 16 | 1.42 | 0.12 |
| Q9VH4         | Cytoplasmic dynein 1 heavy chain 1 | 532 26.7 12 | 2.22 | 0.16  |
| D3YWS7        | Filamin-C               | 289      | 33.3 20.7         | 1.61        | 0.033 * |
Table 1. Cont.

| Accession No. | Protein Name                      | MW (kDa) | iTRAQ Quantitation | Fold Change | p-Value |
|---------------|----------------------------------|----------|--------------------|-------------|---------|
| P80318        | T-complex protein 1 subunit gamma | 61       | 22.7               | 13.3        | 1.7     | 0.0078* |
| P80315        | T-complex protein 1 subunit delta | 58       | 19.3               | 13.3        | 1.45    | 0.016*  |
| Q7TPV4        | Myb-binding protein 1A           | 152      | 21.3               | 11.3        | 1.56    | 0.064   |
| Q6GC67        | Bifunctional glutamate-/proline-tRNA ligase | 170 | 19.7               | 11.3        | 1.74    | 0.033*  |
| Q5265         | ATP synthase subunit alpha, mitochondrial | 60 | 19.3               | 10.3        | 1.87    | 0.17    |
| P2316         | Eukaryotic translation initiation factor 3 subunit A | 162 | 17                 | 11.3        | 1.5     | 0.078   |
| Q7F87         | Staphylococcal nuclease domain-containing protein1 | 102 | 16.7               | 11          | 1.51    | 0.12    |
| B1AX58        | Plastin-3                         | 72       | 16                 | 11.3        | 1.41    | 0.00015*|
| Q9D6N0        | Elongation factor 1-gamma         | 50       | 16.7               | 10.3        | 1.61    | 0.18    |
| Q8BGQ7        | Alanine-tRNA ligase, cytoplasmic   | 107      | 15.7               | 10.3        | 1.51    | 0.21    |
| Q8CGC7        | Fusicin                           | 55       | 15                 | 10.3        | 1.45    | 0.049   |
| Q8CIE6        | Coatomer subunit alpha            | 138      | 14.7               | 10          | 1.47    | 0.025*  |
| P21550        | Beta-elongase                     | 47       | 38                 | 25.3        | 1.5     | 0.002*  |
| Q922F4        | Tubulin beta-6 chain              | 50       | 27.7               | 17.7        | 1.57    | 0.011   |

In the high abundance group of MedC, 50 proteins were down-regulated by more than 50% by RH2 infection (Table 2). They included ECM components such as fibronectin, thrombospondin-1, basement membrane-specific heparin sulfate proteoglycan core protein, fibrillin-1, chondroitin sulfate proteoglycan 4, SPARC, procollagen C, collagen alpha, and extracellular matrix protein 1. Other proteins, cathepsin B, cathepsin D, matrixmetalloproteinase-9, and 72 kDa type IV collagenase, were involved in the remodeling of ECM. Among all proteins listed, a significant difference was observed between the Med24 and MedC groups.

Table 2. Down-regulate proteins in the abundance group of secretomes a, b. a Proteins down-regulated by less than 0.5 fold by the RH2 infection are listed. b Mean of three assessments.

| Accession No. | Protein Name                     | MW (kDa) | iTRAQ Quantitation | Fold Change | p-Value |
|---------------|----------------------------------|----------|--------------------|-------------|---------|
| A0A087WR50    | Fibronectin                       | 263      | 33.7               | 99.0        | 0.34    | 0.00032 |
| P35441        | Thrombospondin-1                  | 130      | 15.7               | 48.3        | 0.32    | 0.00045 |
| E9FZ16        | Basement membrane-specific heparan sulfate proteoglycan core protein | 470 | 9.3                 | 50.0        | 0.19    | 0.011   |
| P97928        | Pigment epithelium-derived factor | 46       | 16.7               | 42.7        | 0.39    | 0.011   |
| A2AQ53        | Fibrillin-1                       | 312      | 10.0               | 41.7        | 0.24    | 0.0000830|
| Q6LC28        | Peroxidase homolog                | 165      | 6.7                | 36.3        | 0.18    | 0.0019  |
| P33434        | 72-kDa type IV collagenase        | 74       | 11.0               | 31.3        | 0.35    | 0.0074  |
| E9FZ70        | Prosaposin                        | 61       | 9.0                | 28.3        | 0.32    | 0.0016  |
| D3Z598        | Latent-transforming growth factor beta-binding protein 4 | 167 | 6.0                 | 29.7        | 0.2     | 0.00022 |
| Q8T797        | Galectin-3-binding protein        | 64       | 7.3                | 25.7        | 0.29    | 0.00022 |
| P28653        | Biglycan                          | 42       | 4.7                | 27.7        | 0.17    | 0.011   |
| P10605        | Cathepsin B                       | 37       | 10.0               | 21.3        | 0.47    | 0.023   |
| Q00493        | Carboxypeptidase E                | 53       | 10.3               | 21.0        | 0.49    | 0.011   |
| Q8V170        | Chondroitin sulfate proteoglycan 4 | 252 | 6.3                 | 25.0        | 0.25    | 0.0015  |
| Q8G699        | Chitinase                         | 52       | 6.3                | 23.3        | 0.27    | 0.00044 |
| Q9R118        | Serum protease HTRA1              | 51       | 7.0                | 22.3        | 0.31    | 0.00078 |
| P07214        | SPARC                             | 34       | 6.3                | 22.7        | 0.28    | 0.00029 |
| Q6Q2T2        | Procollagen C                     | 50       | 4.7                | 24.3        | 0.19    | 0.0000940|
| Q6F0N1        | Adipocyte enhancer-binding protein 1 | 61   | 7.3                 | 19.7        | 0.37    | 0.0078  |
| Q8BND5        | Sulfhydryl oxidase 1              | 83       | 5.3                | 21.0        | 0.25    | 0.0018  |
| Q0219         | Nucleobindin-1                    | 33       | 7.7                | 17.3        | 0.44    | 0.016   |
| Q8R0E2        | Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 | 84 | 5.7                 | 17.7        | 0.32    | 0.0037  |
| Q61468        | Mesothelin                        | 69       | 6.3                | 16.7        | 0.38    | 0.00039 |
| M0QWP1        | Agrin                             | 217      | 2.7                | 18.3        | 0.15    | 0.000003480|
| Q6S508        | Extracellular matrix protein 1    | 63       | 3.0                | 17.7        | 0.17    | 0.0014  |
| O08665        | Semaphorin-3A                     | 89       | 5.3                | 15.0        | 0.36    | 0.0054  |
| Q9WVH9        | Fibrulin-5                        | 50       | 2.3                | 18.0        | 0.13    | 0.00071 |
| Q60693        | Platelet-activating factor        | 49       | 2.3                | 17.7        | 0.13    | 0.00037 |
| O88325        | Alpha-N-acetylglucosaminidase     | 83       | 3.0                | 15.7        | 0.19    | 0.00046 |
| P21460        | Cystatin-C                        | 16       | 5.3                | 13.3        | 0.34    | 0.0058  |
Table 2. Cont.

| Accession No. | Protein Name                  | MW (kDa) | iTRAQ Quantitation b | Fold Change | p-Value |
|---------------|-------------------------------|----------|----------------------|-------------|---------|
| P55065        | Phospholipid transfer protein | 54       | 0.7                  | 18.0        | 0.04    | 0.00048 |
| P25785        | Metalloproteinase inhibitor 2 | 24       | 5.7                  | 12.7        | 0.45    | 0.012   |
| Q6ZQR4        | Out at first protein homolog  | 32       | 4.7                  | 13.7        | 0.34    | 0.0049  |
| G3XA35        | MGC11656, isoform CRA_a       | 263      | 4.7                  | 13.3        | 0.35    | 0.007   |
| Q8CE08        | Prostatic acid phosphatase    | 44       | 2.7                  | 15.3        | 0.17    | 0.000700|
| P41245        | Matrixmetalloproteinase-9     | 81       | 3.0                  | 14.7        | 0.2     | 0.0013  |
| P12023        | Amyloid beta A4 protein       | 87       | 3.3                  | 13.3        | 0.25    | 0.0042  |
| P19242        | Cathepsin D                   | 45       | 5.0                  | 11.7        | 0.43    | 0.034   |
| Q9WV54        | Acid ceramidase               | 45       | 2.7                  | 14.0        | 0.19    | 0.00071 |
| P08121        | Collagen alpha-1 (III) chain  | 139      | 2.0                  | 13.3        | 0.15    | 0.011   |
| G3UWC2        | N-acetylated alpha-linked acidic dipeptidase 2, isoform CRA A | 87 | 1.7 | 11.7 | 0.14 | 0.0013 |
| Q62165        | Dystroglycan                   | 97       | 2.3                  | 10.3        | 0.23    | 0.00043 |
| Q62356        | Follistatin-related protein 1 | 35       | 2.3                  | 10.0        | 0.23    | 0.0019  |
| P11087        | Collagen alpha-1 (I) chain    | 138      | 2.0                  | 10.3        | 0.19    | 0.011   |
| Q6EPL2        | Calsyntenin-3                 | 109      | 0.7                  | 11.3        | 0.06    | 0.0015  |
| Q98620        | Collagen alpha-1 (II) chain   | 184      | 0                    | 12.0        | 0      | 0.00048 |
| Q64857        | Collagen alpha-1 (VI) chain   | 108      | 0                    | 12.0        | 0      | 0.000300|
| Q60195        | Lysosomal alpha-mannosidase   | 115      | 0                    | 10.0        | 0      | 0.000652|
| P42880        | Insulin-like growth factor-biding protein 6 | 25 | 0 | 10.0 | 0 | 0.00098 |

2.2. Alteration of DAMPs and DAMPs-Related Proteins in the Secretomes

In DAMPs, the numbers of high, medium, and low abundance proteins were 11, 6, and 3, respectively (Table 3). In the high abundance group, HSPs, actin cytoplasmic 1, annexin A1, annexin A2, galectin-1, peroxiredoxin-2, and histone H2B type 1 were observed. Only the levels of HSP90-beta, actin cytoplasmic 1, and galectin-3 were significantly higher in Med24 than in MedC. ATP-citrate, which is responsible for the production of ATP, a known DAMP, was also detected as a significantly elevated protein (Table 3).

Table 3. DAMPs and DAMPs-related proteins in secretomes. a Mean of three assessments. * p < 0.05.

| Accession No. | Protein Name                  | MW (kDa) | iTRAQ Quantitation b | Fold Change | p-Value |
|---------------|-------------------------------|----------|----------------------|-------------|---------|
| P63017        | Heat shock cognate 71-kDa protein | 71      | 68.0                  | 63.7        | 1.07    | 0.41     |
| P11499        | Heat shock protein HSP 90-beta | 83       | 66.7                  | 56.7        | 1.18    | 0.046*   |
| P60710        | Actin, cytoplasmic 1          | 42       | 49.0                  | 36.0        | 1.36    | 0.027*   |
| Q3U2G2        | Heat shock 70 kDa protein 4    | 94       | 36.0                  | 30.7        | 1.17    | 0.071    |
| P70901        | Heat shock protein HSP 90-alpha | 85     | 57.7                  | 49.7        | 1.16    | 0.052    |
| P10107        | Annexin A3                   | 39       | 30.0                  | 29.0        | 1.03    | 0.75     |
| P10853        | Histone H2B type 1-F/J/L      | 14       | 27.3                  | 29.3        | 0.93    | 0.71     |
| P63038        | 60-kDa heat shock protein, mitochondrial | 61 | 32.3 | 20.3 | 1.59 | 0.22 |
| P16045        | Galectin-1                   | 15       | 22.3                  | 19.0        | 1.18    | 0.067    |
| P70356        | Annexin A2                   | 39       | 15.3                  | 14.3        | 1.07    | 0.72     |
| Q61171        | Peroxiredoxin -2             | 22       | 15.0                  | 13.7        | 1.10    | 0.33     |
| O08709        | Peroxiredoxin -6             | 25       | 11.7                  | 9.7         | 1.21    | 0.18     |
| Q85816        | Thioredoxin reductase 1, cytoplasmic | 67     | 10.7                  | 8.3         | 1.28    | 0.28     |
| Q61699        | Heat shock protein 105 kDa    | 96       | 12.3                  | 8.0         | 1.54    | 0.25     |
| P16110        | Galectin-3                   | 28       | 10.0                  | 5.7         | 1.67    | 0.031*   |
| P99029        | Peroxiredoxin-5, mitochondrial | 22     | 9.0                   | 6.3         | 1.42    | 0.091    |
| P63158        | High mobility group protein B1 | 25      | 5.7                   | 4.7         | 1.22    | 0.66     |
| P15864        | Histone H1.2                 | 21       | 3.0                   | 7.0         | 0.43    | 0.0081*  |
| P10639        | Thioredoxin                  | 12       | 3.7                   | 3.7         | 1.55    | 0.10     |
| P14211        | Calreticulin                 | 48       | 3.7                   | 3.3         | 1.12    | 0.90     |

DAMPs-related proteins

| Accession No. | Protein Name                  | MW (kDa) | iTRAQ Quantitation b | Fold Change | p-Value |
|---------------|-------------------------------|----------|----------------------|-------------|---------|
| Q6V117        | ATP-citrate synthase          | 121      | 28.0                  | 21.0        | 1.33    | 0.025*   |
| P56480        | ATP synthase subunit beta, mitochondrial | 56 | 24.0 | 17.0 | 1.41 | 0.42 |
| Q6Q265        | ATP synthase subunit alpha, mitochondrial | 60 | 19.3 | 10.3 | 1.87 | 0.17 |
| Q9DR20        | ATP synthase subunit O, mitochondrial | 23 | 4.7 | 4.0 | 1.17 | 0.80 |
2.3. Viral Proteins in the Secretomes

Twenty-nine viral proteins that were detected in Med24 were not present in MedC (Table 4). When viral proteins were categorized into three groups, the numbers of proteins in the high, medium, and low abundance groups were 8, 9, and 12, respectively. Major capsid protein unique sequence of the L component (UL) 19, tegument protein UL37, capsid protein virion polypeptide (VP) 23, glycoprotein D, and glycoprotein E were identified as viral structural proteins. In addition, ribonucleoside-diphosphate reductase, single-strand DNA-binding protein, DNA polymerase processivity subunit, transcriptional regulator infected cell protein (ICP) 4, exonuclease (UL12), multifunctional expression regulator (ICP27), and deoxyuridine triphosphatase were identified as high abundance proteins in Med24 (Table 4).

Table 4. HSV-1 proteins in secretomes. a Mean of three assessments. UL, unique sequence of the L component; US, unique sequence of the S component; ICP, infected cell protein; VP, virion polypeptide.

| Accession No. | Protein Name                                      | MW (kDa) | Gene     | iTRAQ Quantitation | Med24 |
|---------------|---------------------------------------------------|----------|----------|--------------------|-------|
| F8RCG70       | Ribonucleoside-diphosphate reductase large subunit, ICP6 | 124      | UL39     | 33.7               |       |
| D3YP99        | Single-stranded DNA-binding protein, ICP8         | 126      | UL29     | 32.7               |       |
| L0N3H2        | DNA polymerase processivity subunit               | 51       | UL42     | 19.3               |       |
| B9VQC5        | Transcriptional regulator ICP4                    | 133      | α4       | 7.7                |       |
| D3YP92        | Major capsid protein, VP5                         | 149      | UL19     | 14.3               |       |
| A0A0BSE4C5    | UL12, Exonuclease                                | 67       | UL12     | 13.0               |       |
| D3YBP9        | Multifunctional expression regulator, ICP27       | 55       | UL54     | 13.0               |       |
| A0A0F7GRC1    | Deoxyuridine triphosphatase                      | 39       | UL50     | 10.0               |       |
| F8RCGG6       | Tegument protein UL37                            | 121      | UL37     | 10.0               |       |
| A0A0BSE4D5    | Thymidine kinase                                  | 41       | UL23     | 8.3                |       |
| A0A0BSE4F4    | VP13/VP14                                        | 74       | UL47     | 7.7                |       |
| D0V7M2        | Capsid protein VP23                               | 34       | UL18     | 7.7                |       |
| A2I996        | Glycoprotein D                                    | 43       | US6      | 6.7                |       |
| L0N6C7        | Envelope glycoprotein E                           | 59       | US8      | 5.3                |       |
| F8RCR4        | Ribonucleoside-diphosphate reductase small subunit | 38       | UL40     | 6.0                |       |
| F8RDC2        | Tegument protein VP11/12                          | 63       | UL25     | 5.0                |       |
| L0N3F0        | Tegument protein VP22                             | 32       | UL49     | 4.3                |       |
| D3YBR2        | Transactivating tegument protein UL25             | 54       | UL48     | 3.7                |       |
| A0A0F7CY40    | Nuclear egress membrane protein                   | 30       | UL34     | 4.7                |       |
| A0A0F7GTB2    | Capsid maturation protease                        | 67       | UL26     | 1.7                |       |
| A0A0B9YQM2    | Glycoprotein B                                    | 100      | UL27     | 1.7                |       |
| A0A0B9YQK4    | UL2, Uracil-DNA glycosylase                       | 36       | UL2      | 1.3                |       |
| F8RFA5        | Tegument protein UL21                             | 58       | UL21     | 1.3                |       |
| A0A0B9E8E3    | Glycoprotein I                                    | 41       | US7      | 1.0                |       |
| D3YP85        | Envelope glycoprotein H                           | 90       | UL22     | 0.7                |       |
| L0N3E3        | Envelope glycoprotein C                           | 55       | UL44     | 0.7                |       |
| L0N6E6        | DNA polymerase                                    | 137      | UL30     | 0.7                |       |
| A0A0B9WZ14    | Type II membrane protein                          | 18       | UL45     | 0.7                |       |

3. Discussion

Proteomic analysis has been applied to HSV-1 virion-incorporated host proteins, cellular proteins interacting with viral proteins such as ICP27, ICP8, and VP16, and protein profiles in HSV-1-infected cells [17–19]. Miettinen et al. [16] characterized the secretome of HSV-1-infected human primary macrophage using high-throughput quantitative proteomics, and identified 516 distinct proteins from macrophages secretome upon HSV-1 infection, and the secretion of 411 proteins was >2-fold increased upon interferon β (IFN-β) priming and/or HSV-1 infection. Based on our previous findings that the supernatants of RH2-infected cells suppressed the tumor growth in mice [13], we hypothesized that the supernatants contained immunostimulatory proteins, and performed proteomic analysis of the secretomes. Since the amounts of proteins detectable in the secretomes were important, we classified MedC proteins into three categories based on the results of iTRAQ quantitation, and then determined whether they were up-regulated or down-regulated by RH2 infection. When the high abundance proteins that increased by more than 1.4 fold following RH2 infection were selected, 29 proteins
fulfilled this criterion. However, a significant increase was only observed in 16 proteins, including filamin alpha, tubulin beta-4B, clathrin heavy chain, and TCP-1s (Table 1).

Actin-binding proteins, filamin and pastin-3, and the actin-bundling protein, fascin, were identified as up-regulated high abundance proteins. An increase in tubulin, which is involved in the polymerization of microtubules, was demonstrated. Autoantibodies against filamin C were elevated in the patients with low-grade glioma and those against tubulin increased in the patients with nasopharyngeal carcinoma and neuroblastoma [20–22]. Clathrin heavy chain is one of the most important proteins known to be involved in the secretion and transport of vesicles [23]. Seliger et al. [24] examined potential candidate biomarkers and novel targets for T-cell-based immunotherapies of renal carcinoma, and identified human leukocyte antigen class I ligands, including clathrin heavy chain. Filamin, tubulin, and clathrin may be potential immunogenic antigens in specific cancers.

We also identified TCP-1 family members as the major up-regulated proteins in Med24 secretomes. Chaperonin-containing TCP-1 (CCT), known as the TCP-1 ring complex (TRiC), forms an oligomer that consists of distinct subunits (alpha, beta, gamma, delta, epsilon, zeta, eta, and theta) occupying a fixed position within the two back-to-back chaperonin rings [25]. CCT was originally identified as a molecular chaperone that is required for the folding of the highly abundant cytoskeletal proteins, actin and tubulin [26]. The genes for CCT alpha and CCT beta were amplified in breast cancer and necessary for cancer growth and proliferation [27]. CCT beta and CCT epsilon were overexpressed in both hepatocellular and colorectal cancers [28,29]. Gao et al. [30] reported that CCT5 (CCT epsilon) induced an autoantibody response in non-small cell lung cancer sera, and showed higher expression in cancer tissues. In the case of melanoma immunotherapy, mutated TCP 1-zeta-6A (CCT6A) antigen had previously been identified and proved to be immunogenic in a melanoma patient, so mutated CCT6A peptide was used as a model for patient-specific neoantigen [31]. Therefore, TCP-1 subunits identified in the present study may act as TAAs required for capable of activating tumor-specific immunity.

In secretomes of HSV-1-infected macrophages, endogenous danger signal proteins were demonstrated as up-regulated proteins. They included HSPs, annexins, S100-A proteins, galectins, and thioredoxin superfamily members [16]. The HSP family comprises proteins that act as molecular chaperones and catalyze the folding of proteins. HSPs bind to potential antigens under necrotic conditions and deliver them to various antigen-presenting cells [32]. We found 20 proteins that are known as DAMPs (Table 3). High abundance DAMPs including heat shock cognate 71 kDa protein, HSP90-alpha, HSP 70 kDa protein 4, HSP60, annexin A1, and peroxiredoxin-2 increased by RH2 infection, but the fold change observed was less than 1.4 fold. The levels of HSP90-beta, actin cytoplasmic 1, and galectin-3 were significantly higher in Med24 than in MedC. ATP is a known DAMP released extensively during ICD [4]. We also confirmed that ATP-citrate synthase and ATP synthase were up-regulated proteins as DAMPs-related proteins. The spatiotemporally coordinated emission of specific DAMPs promotes the recruitment of antigen presenting cells to sites of ongoing ICD, their ability to take up dead cell-derived particulate materials, as well as their capacity to prime an adaptive immune response [33]. Up-regulated DAMPs and DAMPs-related proteins observed in the present study were considered to be involved in the augmentation of antitumor immunity, operating as adjuvants.

Exosomes are small membrane-derived vesicles secreted by many types of normal and tumor cells [34]. Xiang et al. [35] reported the presence of DAMPs, such as HSPs, annexins, and histones, in exosomes, indicating the role of microvesicles as a form of excretion. DAMPs observed in the secretomes of MedC may be partly contained in exosomes. However, since oncolytic viruses including RH2 have been shown to induce apoptosis, necroptosis, autophagy, and pyroptosis [3,5,36–38], increases in the secretion of DAMPs may be mostly due to the disintegration of the cell membrane by virus-mediated cell death.

Another result of the present study is the marked decrease in the release of proteins constituting the tumor microenvironment (TME). In the present study, 50 proteins were selected as down-regulated
proteins that were reduced to less than 0.5 (Table 2). They included fibronectin, thrombospondin-1, basement membrane-specific heparan sulfate proteoglycan core protein, fibrillin-1, 72 kDa type IV collagenase, procollagen C, and extracellular matrix protein 1. Since tumor and tumor stroma create the TME, which efficiently promotes tumor progression and supports evasion from antitumor immunity [39,40], oncolytic HSV-1 may exert its antitumor effects by decreasing the release of ECM components.

Using the secretomes of HSV-1-infected macrophages, 5 envelope proteins, 3 tegument proteins, alkaline nuclease, and deoxyuridine 5′-triphosphate nucleotidohydrolase were identified [16]. In the present study, viral structural components, i.e., the tegument, nucleocapsid, and envelope, were also detected in the supernatants of HSV-1-infected SCCVII cells. Furthermore, viral enzymes responsible for viral replication such as ribonucleoside-diphosphate reductase, single-strand DNA-binding protein, DNA polymerase processivity subunit, deoxyuridine triphosphatase, and thymidine kinase were identified. This result indicated that these cytoplasmic and nuclear viral proteins were passively released from dying cells. These proteins have been suggested to act as PAMPs, which promote inflammatory reactions and induce the infiltration of innate and adaptive immune cells.

Immunological abnormalities associated with TME inhibit the priming of antitumor adaptive immunity or tolerize tumor-specific CD4+ and CD8+ T cells [40,41] (Figure 1A). Oncolytic virotherapy is supposed to release DAMPs, PAMPs, and TAA triggering proinflammatory cytokine release, DC maturation, and cytotoxic CD8+ T cell proliferation, and overcome TME-associated immunosuppression by reducing secretion of stromal components [5,40,42,43] (Figure 1B). Using the supernatants of RH2-infected SCC cells, we herein demonstrated that DAMPs increased, and ECM proteins decreased. Viral gene products were also included. This may promote the immunological effects of the oncolytic HSV-1 on SCC cells. As a next step, it is important to confirm the role of each secreted protein in the antitumor effects of oncolytic HSV-1 by specific deletion.

**Figure 1.** A model of the augmentation of antitumor immunity by an oncolytic virus. (A) Immunological abnormalities associated with TME inhibit the priming of antitumor adaptive immunity or tolerize tumor-specific CD4+ and CD8+ T cells; (B) Oncolytic viruses induce ICD with the release of secretomes containing DAMPs, PAMPs, proinflammatory cytokines, and TAA. This triggers DC maturation and cytotoxic CD8+ T cell proliferation. The reduced secretion of stromal components due to viral infection also overcomes TME-associated immunosuppression. TME, tumor microenvironment; ECM, extracellular matrix; TAA, tumor-associated antigen; ICD, immunogenic cell death; DC, dendritic cell; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns.
4. Materials and Methods

4.1. Cells and Virus

SCCVII cells derived from the cutaneous SCC of C3H mice were cultured with Eagle’s minimal essential medium supplemented with 10% calf serum at 37 °C in a humidified atmosphere with 5% CO₂ [13,14]. HSV-1 RH2 lacking the γ34.5 gene and with multiple mutations including glycoprotein B were grown in Vero cell monolayers and infectivity was assessed using a plaque assay on a Vero cell monolayer [14,44].

4.2. Preparation of Concentrated Supernatants of RH2-Infected Cells

SCCVII cells were infected with RH2 at a multiplicity of infection of 10. After being incubated for 60 min for virus adsorption, cell monolayers were washed twice with phosphate-buffered saline, covered with serum-free medium, and cultured for 24 h. Culture supernatants were then harvested, and centrifuged at 1500 rpm for 10 min to remove cell debris. The supernatants were concentrated 30 times using Amicon® Ultra-15 3 K Centrifugal Filter Devices (Merck, Darmstadt, Germany) [13]. Concentrates were exposed to ultraviolet irradiation at an intensity of 0.15 mW/cm² for 30 min in order to inactivate the infectivity of the virus, filtered through a 0.20-µm filter, and named Med24. Mock-infected cells were also incubated in serum-free medium, supernatants were harvested 24 h later, treated as described for RH2-infected cells, and named MedC.

4.3. Proteomic Analysis

Three samples were used each in the proteomic analysis of Med24 and MedC [45]. Protein concentrations in Med24 and MedC were assessed, and 4 volumes of cold acetone per 100 µg were added to each sample. They were kept at −20 °C overnight, and centrifuged at 15,000 rpm for 10 min to collect precipitates. Proteins were dried, and denatured by resuspending in 20 µL of lysis buffer (50 mM Tris-HCl, pH 9.0, 6 M uric acid, and 5% sodium deoxycholate), and reduced with 2 µL of reduction buffer (10 mM dithiothreitol) at 37 °C for 1 h. In order to block cysteine, 1 µL of cysteine-blocking buffer (55 mM iodoacetamide) was added, and proteins were alkylated in the dark at 25 °C for 30 min. After a 10-fold dilution of samples with 50 mM Tris-HCl (pH 9.0), proteins were disintegrated with trypsin at 37 °C for 16 h. An equal volume of ethyl acetate was added and acidified to a final concentration of 0.5% trifluoroacetic acid. The aqueous solution after centrifugation at 14,000 rpm for 2 min was harvested. Samples were desalted on C18-Stage Tips and labeled with the iTRAQ tag dissolved in 70 µL ethanol at room temperature for 1 h.

In the LC-MS/MS analysis, the Ultimate 3000 Nano LC system (Thermo Fisher Scientific, Waltham, MA, USA) was used for ultra performance liquid chromatography (UPLC) and connected to the Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) with a nano-electrospray ionization source. Labeled samples were injected into UPLC and concentrated in a C18 reverse phase trap column (100 µm I.D. × 5 mm length, Thermo Fisher Scientific) at a flow rate of 4 µL/min. Samples were then separated using a C18 reversed phase column (75 µm I.D. × 150 mm long, Nikkiso Tecnos, Tokyo) at a flow rate of 300 nL/min. A fixed mobile phase condition to Solvent A was water containing 0.1% formic acid and to Solvent B was acetonitrile containing 0.1% formic acid. Peptides were ionized by the positive mode of nano-electrospray ionization, which was a 1.8-kV capillary voltage.

Protein data were analyzed using Mascot Distiller v2.4 (Matrix Science, London, UK) http://www.matrixscience.com/mascot_support_v2_4.html, and a list of peaks was generated based on the recorded fragmentation spectra. In the identification of this protein, the UniProt amino acid sequence database (released on 4 March 2015: http://www.uniprot.org/) with biological species limited to the mouse (NCBI Taxonomy ID: 10090) and herpesviridae (ID: 10292) was used. The identified proteins were checked using the UniProt-GOA database (v140, http://www.ebi.ac.uk/GOA), and the annotation of Gene Ontology was added. Qualitative data and quantitative values were assessed using
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Mascot v2.4. In protein identification, the criteria of false rates of detection were less than 1%. The mass error was 10 ppm in Precursor Mass, and 0.01 Da in Fragment Mass. Due to the semi-quantitative nature of this approach, proteins were classified into three categories based on the values of iTRAQ quantitation (<5, low abundance; 5 to 10, medium abundance; and >10, high abundance) [46].

4.4. Statistical Analysis

Statistical analyses were performed using the Student’s t-test with Microsoft Excel (Microsoft, Redmond, WA, USA). Results were expressed as the mean ± SD. Differences were considered to be significant at \( p < 0.05 \).

5. Conclusions

Our results indicate that a HSV-1 RH2 infection increases the release of danger signal proteins and viral gene products, but decreases the release of ECM components. These changes may alter the tumor microenvironment and promote anti-tumor immunity against SCC.

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